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Exploring environmental factors and gene-environment interactions in Autism Spectrum Disorder: a pilot study

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"Just like moons and like suns,
With the certainty of tides,
Just like hopes springing high,
Still I'll rise."
Maya Angelou

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ABSTRACT

Autism Spectrum Disorder (ASD) is a pervasive and clinically heterogeneous neurodevelopmental disorder by deficits in social communication and interactions skills, and repetitive and stereotyped behaviours. It has becomes apparent that ASD has a strong genetics component. However, the level of heritability is still debated: compared to older and smaller studies, results from recent studies estimate a lower heritability percentage (83%), thus leaving a meaningful percentage of the risk that could be explained by environmental factors. Exposure to potentially harmful environmental factors can result in neurodevelopmental issues, due to neurotoxicity. Such environmental factors include endocrine disruptive chemicals like BPA, PBDEs, phthalates, PAHs, pesticides, and heavy metals, which disrupts optimal hormonal function, as well as pharmaceutical drugs which are able to cross physiological barriers and come into contact with the fetus. Moreover, the incorrect metabolization of these xenobiotics due to defective enzymatic activity may be linked to an increased risk of ASD. It has been suggested that individuals carrying polymorphisms that hinder the activity of cytochrome p450 enzymes, responsible for phase I metabolism, as well as UDP-glucuronosyltransferases and glutathione S-transferase enzymes responsible for phase II metabolism, will be more susceptible to potentially toxic chemicals. Therefore, considering the role played by environmental factors, this pilot study aims to investigate and contribute to the understanding of gene-environment interactions in ASD. In order to do this, we explored the usage of the Early Life Exposure Assessment Tool (ELEAT): a questionnaire which indirectly examines child's exposure to exogenous factors that could be related to ASD from 3 months before conception, during pregnancy, to the first year of the child's life, by analyzing maternal exposure. We aimed to investigate the type of data that could be obtained from this questionnaire, how it could be treated, and ultimately how it could be related to genetic information obtained from probands. The questionnaire was filled by 20 Portuguese mothers who had been part of a previous study. Additionally, we were able to obtain 14 biological samples from the children whose mothers filled out the questionnaire, who were then screened for various functional polymorphisms in genes known to interact with the environmental factors investigated in ELEAT. Finally, we related probands' genotype to exposure reported in the ELEAT. Our results suggest that mothers were indeed exposed to some of the environmental factors being studied by the ELEAT in these critical periods of neurodevelopment. Additionally, genotyping results showed that this group of probands did carry a number of the polymorphisms being investigated in this pilot study, which could make them more susceptible to certain xenobiotics. When we merged probands' genotype to reported exposure, we concluded that probands may have been exposed to the chemicals they were sensitive to during neurodevelopment. Most of the variants investigated in this study have not been yet related to ASD, but have the potential to indirectly contribute to the disorder's onset. Our results demonstrate that meaningful results can be obtained from combining the ELEAT with genetic information. Seeing as the general population is somewhat regularly exposed to some levels of these chemicals, genetic factors play a crucial role in increasing susceptibility and thus leading to negative consequences such as increased ASD risk. This pilot study is an important step for future studies that intend to use the ELEAT to identify environmental risk factors for ASD. These should include a large population sample, and an equal number of controls, in order to obtain statistical power when calculating the multiplicative effect of given environmental exposures with genetic liability. In conclusion, the ELEAT could play a vital role in the understanding of gene-environment interactions, and the development of preventive strategies for autism.

Key words: *autism, environment, genetics, metabolism, detoxification*

RESUMO ALARGADO

Introdução: A Perturbação do Espectro do Autismo (PEA) é uma perturbação do neurodesenvolvimento clinicamente heterogénea caracterizada por défices na capacidade de comunicação e interação social, interesses repetitivos e comportamentos estereotipados. A PEA inclui indivíduos que possuem desde baixo até alto funcionamento e pode apresentar-se acompanhada de um vasto número de co-morbilidades, como a Síndrome do X Frágil, epilepsia e problemas gastrointestinais, que contribuem para a sua heterogeneidade fenotípica. A PEA tem uma forte componente genética, com estudos recentes a estimarem uma heritabilidade na ordem dos 83%. Estes valores sugerem também, que uma parte do risco seja explicada por causas não-genéticas, nomeadamente a presença de concentrações séricas baixas de certos fatores nutricionais como a vitamina D e o ácido fólico já foram relacionados com o aumento do risco de desenvolver a perturbação. Também a exposição a tóxicos ambientais, potencialmente prejudiciais e neurotóxicos, pode resultar em problemas no neurodesenvolvimento, Estes incluem: disruptores endócrinos como o bisfenol A (BPA), éteres de difenila polibromados (PBDEs), ftalatos, hidrocarbonetos aromáticos policíclicos (PAHs), pesticidas e metais pesados (nomeadamente chumbo e mercúrio), que interferem com o funcionamento hormonal normal, bem como medicamentos capazes de atravessar as barreiras fisiológicas (hematoencefálica e placentária) e interferir com o feto. Estudos recentes suportam, com diferentes níveis de evidência, a associação entre a exposição a xenobióticos e o risco de desenvolver PEA. Tem sido sugerido que indivíduos portadores de polimorfismos que alteram a atividade das enzimas do citocromo p450, responsáveis pelo metabolismo de fase I, bem como as enzimas UDP-glucuronosiltransferases e glutationa S-transferases, responsáveis pelo metabolismo de fase II, serão mais suscetíveis a substâncias químicas potencialmente tóxicas. Normalmente, os estudos na PEA tendem a focar-se somente em fatores genéticos ou em fatores não-genéticos, havendo uma necessidade urgente de desenvolver abordagens que integrem conjuntamente as duas componentes.

Objetivo: Considerando que alguns casos de PEA poderão resultar da combinação entre a suscetibilidade genética individual e a exposição a fatores ambientais durante o neurodesenvolvimento, com este estudo piloto pretende-se investigar e contribuir para um melhor conhecimento das interações gene-ambiente na etiologia da PEA.

Métodos: Foi explorada a aplicação do instrumento de investigação denominado: *Estudo Longitudinal da Exposição Ambiental a Toxinas* (ELEAT) - um questionário detalhado que examina indiretamente a exposição das crianças a fatores exógenos que podem estar relacionados com a PEA, desde os 3 meses antes da conceção, no decurso da gestação e estendendo-se ao longo do primeiro ano de vida. Nesta fase pretendemos investigar que tipo de dados podem ser obtidos a partir deste questionário, o modo como os dados podem ser tratados e, finalmente, como podem ser relacionados com a informação genética subsequentemente obtida de probandos. Responderam ao questionário 20 mães portuguesas com pelo menos um filho com PEA, recrutadas através de um estudo anterior. Na avaliação da componente genética do estudo, 14 amostras de DNA de crianças com PEA (cujas mães preencheram o questionário) foram testadas para a presença de vários polimorfismos funcionais em 14 genes: *ABCB1, ACHE, AHR, CYP2D6, CYP2C19, CYP3A4, GSTM1, MTHFR, PLCG1, PON1, TNFRSF11B, UGT1A, UGT2B15, VDR*. Este conjunto de genes foi selecionado pela sua relação com os fatores ambientais investigados no ELEAT, e os polimorfismos investigados têm potencial de influenciar a forma como os indivíduos portadores respondem à exposição a esses fatores. Finalmente, relacionámos o genótipo dos probandos com a exposição a xenobióticos reportada no referido questionário.

Resultados: Os resultados obtidos do ELEAT sugerem que as mães e respetivos filhos(as) foram expostos a alguns dos fatores ambientais estudados, durante períodos críticos para o neurodesenvolvimento. Os resultados da genotipagem mostraram que o grupo de probandos é portador de variantes em vários dos genes investigados neste estudo, o que poderá torná-los mais suscetíveis a certos xenobióticos. Foram

identificados portadores de alelos de menor frequência em genes como o *ABCB1, ACHE, AHR, CYP2R1, CYP2C19, CYP3A4, PON1, UGT2B15* e *VDR.* Foram também encontrados indivíduos homozigóticos para o alelo alternativo em polimorfismos dos genes *ACHE*, *AHR, PON1* e *UGT2B15*. Estes genes são particularmente importantes na resposta à exposição a pesticidas, PAHs e BPA, o que poderá ter um impacto na forma como os seus portadores respondem a tais exposições. Indivíduos com estes genótipos poderão exibir um maior risco de desenvolvimento de problemas no neurodesenvolvimento, como a PEA. Quando cruzamos o genótipo dos probandos com a exposição reportada, verificamos que os probandos portadores de alguns dos polimorfismos podem ter sido expostos aos produtos químicos aos quais são sensíveis durante períodos críticos do desenvolvimento neurológico.

Discussão: Os nossos resultados demonstram que se podem obter dados importantes da combinação do *ELEAT* com informação genética. Apesar de extenso, o questionário relevou-se uma ferramenta útil para a recolha de informação relativa à exposição ambiental. A genotipagem de vários polimorfismos comuns na população geral, mas que poderão afetar a metabolização de vários fatores ambientais, poderá constituir uma abordagem apropriada para a identificação de indivíduos em maior risco de desenvolver PEA. Uma vez que a população geral está regularmente exposta a alguns dos produtos químicos analisados, os fatores genéticos desempenham um papel crucial no aumento da suscetibilidade e, portanto, podem levar a consequências negativas, como o aumento do risco a PEA. Algumas variantes investigadas neste estudo ainda não foram relacionadas com a PEA, mas têm o potencial de contribuir indiretamente para o desenvolvimento da patologia. Este estudo piloto é um passo importante para futuros trabalhos de investigação que pretendam usar o ELEAT para identificar fatores de risco ambientais para a PEA. Tais estudos deverão incluir uma amostra populacional grande e igual número de controlos, a fim de obter poder valor estatístico para calcular o efeito combinado de exposições ambientais e suscetibilidade genética.

Conclusão: Apesar da sua limitada dimensão, o estudo aqui apresentado é de particular relevância pois é um ponto de partida para o estudo conjunto de fatores genéticos e não-genéticos na PEA. Dado que o contacto com fatores ambientais poderá ser modificado de forma a evitar a exposição por indivíduos particularmente suscetíveis, o estudo de fatores de risco ambientais é de extrema importância. Em conclusão, a integração de informação recolhida através do ELEAT com informação genética poderá contribuir para a compreensão das interações gene-ambiente e para o desenvolvimento de estratégias preventivas para a Perturbação do Espetro do Autismo.

Palavras-chave: *autismo , ambiente, genética, metabolismo, destoxificação*

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ABBREVIATIONS

ADHD – Attention Deficit Hyperactivity Disorder
ASD – Autism Spectrum Disorder
ASDEU – Autism Spectrum Disorder in the European Union
BBB – Blood-Brain Barrier
BPA – Bisphenol A
BSA – Bovine Serum Albumin
CDCV – Common Disease Common Variant
CNS – Central Nervous System
CNV – Copy Number Variation
DNA – Deoxyribonucleic Acid
DPS – Health Promotion Department
DSM – Diagnosis and Statistical Manual of Mental Disorders
EDCs – Endocrine Disruptive Chemicals
EDTA – Ethylenediaminetetraacetic Acid
ELEAT – The Early Life Exposure Assessment Tool
EM – Extensive Metabolizer
FPE – Female Protective Effect
IM – Intermediate Metabolizer
INSA – National Health Institute Doutor Ricardo Jorge
IQ – Intelligence Quotient
OC – Organochlorine Pesticides
OP – Organophosphate Pesticides
PBB – Polybrominated Biphenyl
PBDE – Polybrominated Diphenyl Ethers
PCB – Polychlorinated Biphenyl
PCR – Polymerase Chain Reaction
PDDNOS – pervasive developmental disorder not otherwise specified
PM – Poor Metabolizers
ROS – Reactive Oxygen Species
SNP – Single Nucleotide Polymorphism
SNV – Single Nucleotide Variant
TBE – Tris/Borate/EDTA
UEM – Ultra-Extensive Metabolizer
UGT – Glucuronosyltransferas

1. INTRODUCTION

1.1 AUTISM SPECTRUM DISORDER

Autism Spectrum Disorder (ASD) is a pervasive and clinically heterogeneous neurodevelopmental disorder characterized by deficits in social communication and interactions skills, and repetitive and stereotyped behaviours. Individuals with ASD may be overly dependent on routines, highly sensitive to changes in their environment, or intensely focused on unusual item parts (American Psychiatric Association, 2013). The symptoms vary from person to person, ranging from high-functioning to low-functioning individuals. One of the important changes in the fifth edition of the Diagnosis and Statistical Manual of Mental Disorders (DSM-5) is that the subgroups in Autism Disorder presented in DSM-4 were merged (Kulage, Smaldone, & Cohn, 2014). Therefore, the term ASD is currently used to define a broad concept of autism, manifested as a spectrum of behavioral, cognitive, and linguistic problems that include autistic disorder, Asperger syndrome, and pervasive developmental disorder not otherwise specified (PDDNOS) (American Psychiatric Association, 2013).

ASD is usually diagnosed during early childhood, around 2 to 3 years of age. Early symptoms such as failure to respond to one's name, reduced interest in social interaction and delayed babbling are common. One of the key features that hinders social interactions is the difficulty of seeing things from another person's perspective, and therefore, difficulties in interpreting social cues (What Are the Symptoms of Autism? 2018; American Psychiatric Association, 2013).

ASD occurs in all social, racial, ethnic and socioeconomic groups, with a prevalence of about 10 out of 10,000 children in Portugal (Oliveira et al., 2007). In terms of global prevalence, studies in different countries estimate that the median ASD prevalence is of 17/10 000 (Elsabbagh et al., 2012). Higher prevalence rates in boys compared to girls are consistently observed, with a male to female ratio of 4:1 (Werling & Geschwind, 2013).

This disorder has significant caregiver, family and financial burdens, and lifelong impact to the affected individuals (Van Heijst & Geurts, 2015). There is no treatment for autism, but as children grow up and undergo therapy, symptoms may lessen and become milder. Early behavioral therapies are recommended as these have been shown to have more benefits. Intervention can involve behavioral treatments, medicines or both. ASD often appears with other comorbidities, such as seizures, intellectual disability, attention deficit hyperactivity disorder, and gastrointestinal distress. Addressing these conditions can improve attention, learning and related behaviors (American Psychiatric Association, 2013).

ASD is a very complex disorder, and despite much research, its cause is still not evident. In the past, studies have mostly focused on the role of genetic factors, but it is becoming more apparent that non-genetic factors are substantial players too. Since genetic factors alone are not sufficient to explain autism itself, it is now suggested that environmental factors may modulate ASD risk in individuals who are genetically susceptible.

1.2 GENETICS & HERITABILITY

The initial heritability estimates for ASD were very high (Sandin et al., 2017), prompting many research groups to search ASD genes. Nowadays, ASD is recognized to be genetically heterogeneous, therefore making it a complex disorder to study. However, technology advances have allowed for substantial progress in gene discovery. Common disease common variant (CDCV) used to be the dominant theory held in genetic studies of ASD. It was a common belief that, since most living humans come from the result of a rapid expansion of the human population, they share a substantial amount of genetic variation called common variants (>1%). Accumulation of common variants of low effect is the likely explanation for a majority of ASD cause. In other words, common variants are present in all human populations and control for normal phenotypes, but in particular combinations, they may result in the abnormal phenotypes of ASD, therefore increasing social and language dysfunctions. On the other hand, recent results from genetic investigations have shown the important contribution of rare genetic variants (<1%) in addition to common variants (Geschwind & State, 2015).

The study of rare and *de novo* variants has brought about a dramatic progress in ASD genetic research during the past decade. The Human Gene Module from the Simons Foundation Autism Research Initiative (SFARI), an online database that includes a large collection of genes implicated in autism. Genes are divided into different categories depending on their scores. Currently, high confidence syndromic genes, with category 1 score, include: *ADNP*, *AIRD1B*, *ASXL3*, *CHD2*, *CHD8*, *DYRK1A*, *KMT2A*, *NAA15*, *POGZ* and *SHANK3*, which contain rare genetic variants. Variants found in ASD subjects include: *de novo* frameshift variants in the *ADNP* gene, de novo loss of function variant and a missense variants in the *NAA15* gene, *de novo* deletions within the *ARID1B* gene resulting in reduced transcript expression, and *de novo* loss of function variants in the *DYRK1A* gene. Additionally, other genes in the database such as *MTF1*, *ESR1*, *ADORA2A*, *SHANK3* among others are also targeted by common variants. This reinforced the importance of studying both types of variants in ASD (SFARI Gene, 2018).

On account of different heritability studies, it has become apparent that ASD onset could be due to several different genetic factors variations. That is, that a proportion of ASD phenotype variance can be explained by genetic factors. However, the level of heritability is still debated. A recent twin study performed in Sweden estimated that ASD heritability was 83% (Sandin et al., 2017). This estimate is higher than that of the California twin study, where the authors estimated a genetic heritability of 38%, and a 58% estimate for shared environmental factors (Hallmayer et al., 2011). These results lead the researchers to conclude that, although genetic factors also play an important role in ASD, a significant proportion of the variance in liability can be explained by environmental factors common to twins (Hallmayer et al., 2011). This study underlines the importance of non-genetic environment factors in the development of ASD. Although, during the years, different studies have put ASD heritability on the range of 38% to 90%, there is still a meaningful percentage that can be explained by environmental factors (Sandin et al., 2017).

1.3 Environmental Factors and Autism Spectrum Disorder

Since there has been an accumulation of evidence pointing to the role played by environmental factors in ASD risk, a considerable number of studies have focused on this area, which was previously underexplored. As seen in figure 1, there are various mechanisms through which certain environmental factors can affect one's genome to increase the risk of ASD onset.

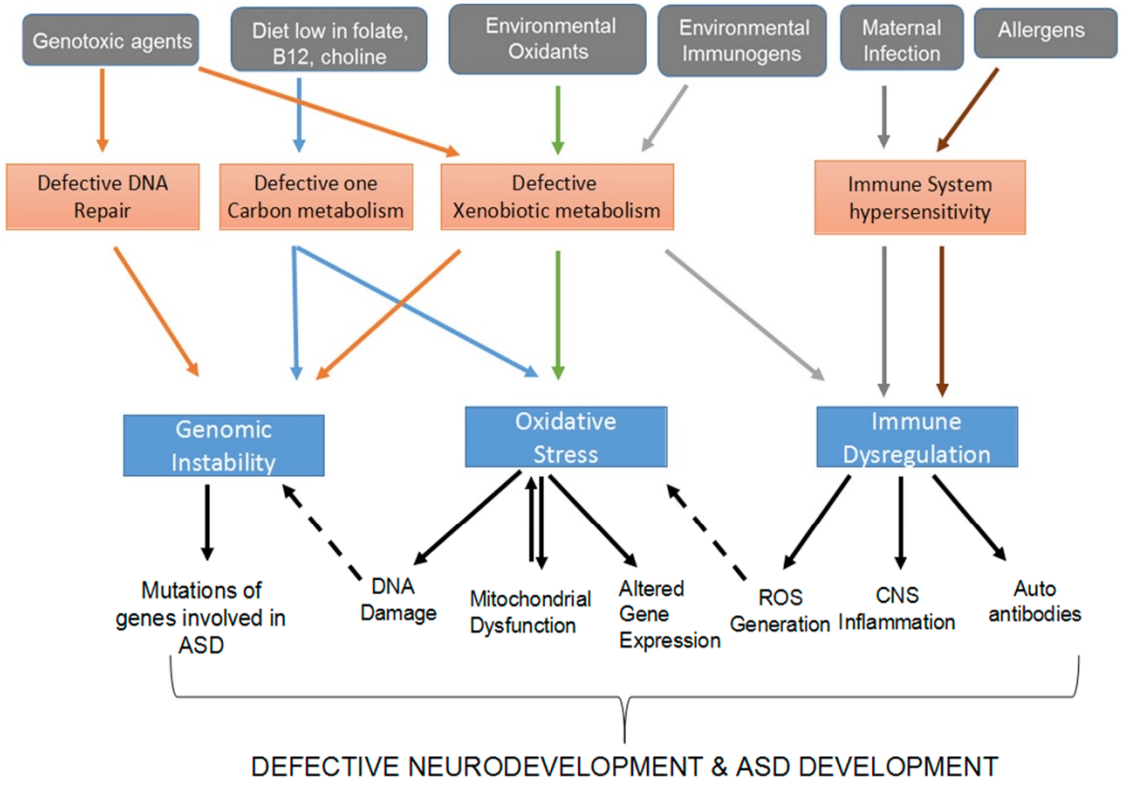


Figure 1: Interactions between environmental and genetic factors that modulate the genome and epigenome of susceptible individuals. The grey boxes present environmental factors, the orange boxes present genetic factors. The blue boxes represent biological mechanisms. (adapted from Koufaris & Sismani 2015)

When mechanisms responsible for body homeostasis are debilitated, the interaction with potentially hazardous environmental factors may result in internal dysregulation such as abnormal immune activation, increased oxidative stress, as well as genomic instability. It has been established that immune system dysregulation, including infections in early pregnancy, prenatal maternal inflammation, and increased neuronal inflammation, could be contributing factors for the development of ASD. This could be due to the fact that chronic inflammation can be a source of reactive oxygen species (ROS), thus contributing to oxidative stress. Moreover, because imbalanced cytokine release can disrupt neurophysiological neurodevelopment due to their ability to affect both development and function of the neuronal system. Factors that can trigger these immune responses include environmental exposures to toxins, allergens and infections, as well as failure from mechanisms that clear these xenobiotics from the body, such as metabolic enzymes (Koufaris & Sismani, 2015).

Studies have also shown that environmental factors have the ability to affect epigenics. These are mechanisms that biochemically modify DNA or histones affecting gene expression, without changing the

DNA sequence (Modabbernia, Velthorst, & Reichenberg, 2017). Environmental factors associated with ASD that can affect these mechanisms include exposure to heavy metals, levels of dietary folate (Koufaris & Sismani, 2015), as well as maternal use of valproate, used primarily to treat epilepsy and bipolar disorders, due to its ability to inhibit histone deacetylase and interference with folic acid metabolism (Modabbernia et al., 2017).

1.3.1 Endocrine Disruptive Chemicals

A category of environmental factors known to dysregulate optimal bodily functions, and that have been linked to the risk of ASD development are Endocrine Disruptive Chemicals (EDCs). According to the U.S Environmental Protection Agency, an EDC is "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process." (Diamanti-Kandarakis et al., 2009). These substances can be either natural or synthetic, and result in modifying the systems that enable the organism to communicate with and respond to the environment. They affect: nuclear receptors, non-nuclear steroid hormone receptors, nonsteroid receptors (e.g.: neurotransmitters), and enzyme pathways (Diamanti-Kandarakis et al., 2009). ASD may be associated with increased sensitivity to EDCs, where vulnerability depends on one's genotype: individuals who are more susceptible could have more serious reactions when exposed even to low doses of EDCs, which could result in increased risk of ASD due to abnormalities in brain development and in hormone levels (Koufaris & Sismani, 2015).

Examples of EDCs include plastics (bisphenol A), plasticizers (phthalates), pesticides (DDT, chlorpyrifos), fungicides (vinclozolin), pharmaceutical agents, heavy metals, and chemicals used as industrial solvents/lubricants and their byproducts (PCBs, PBB, PBDEs) (Diamanti-Kandarakis et al., 2009). Bisphenol A (BPA) is used in a wide variety of applications such as food-can lining and baby bottles, while alkylphenols, another type of phenol, is used products such as detergents and pesticide formulations (Yi et al., 2013). Due to its disruptive effects, the use of BPA in baby bottles has been banned in some countries, but it can still be found in alternative sources. Persistent Organic Pollutants, PCBs, are synthetic carbon-based chemicals that accumulate in the environment and in human tissues. Diet is their main source of exposure due to their ability to bioaccumulate in predatory fish and breast milk (Kalkbrenner, Schmidt, & Penlesky, 2014). There is a wide range of pesticides available in the market, characterized by different functions, such as insecticide, herbicide, fungicide, among others. Whereas in general they are all known to have toxic effects on humans; organophosphates (OP) and organochlorines (OC) have the most evidence of neurotoxicity, and therefore, are thought to increase the risk of ASD development. OC pesticides have been recognized to interfere with neurodevelopment due to their EDC properties, while OP pesticides restrict neurotransmission in both peripheral and central nervous system (CNS) by inhibiting acetylycholinesterase production in the brain, thus affecting synapse formation, axon transmission, cell maturation, and programmed cell death. Heavy metals such as lead and mercury have highly significant and consistent evidence of hindering neurodevelopment, resulting in damages such as the loss of IQ points and behavioral problems. The main source of exposure for lead is via inhalation, as a consequence of its diffusion into the air from the usage of leaded gasoline and paints, and via ingestion for mercury, due to the bioaccumulation of methylmercury in food (Kalkbrenner, Schmidt, & Penlesky, 2014).

Endocrine disrupting chemicals are also known to target neurotransmitters such as dopamine, norepinephrine, serotonin, and glutamate. This is important because it explains effects of EDCs on cognition, learning, memory, and other behaviors which can be related to ASD (Diamanti-Kandarakis et al., 2009). Twin studies have found that variation in alleles of the serotonin transporter gene at *17q11–q12* are more frequent in individuals with autism (Tordjman et al., 2014). Following that these variants in individuals with ASD may attribute an increased sensibility to EDCs, which are known to target neurotransmitters such as serotonin, it could be predicted that their reaction to EDC exposure could be even more detrimental than that of an individual without such genetic variants.

The role played by sex hormones in the development of ASD have been a subject of study as an attempt to determine the source of sex differences in autism. Due to the action of hormones during embryonic and early postnatal development, there is the masculinization of the brain through the presence of estradiol & testosterone, and feminization of the brain through the absence of steroid hormones for females in this period. Research has shown that PCBs, phytoestrogens, fungicides, pesticides, and other xenobiotics can disrupt brain sexual differentiation. When these disruptions happen during critical periods, they can lead to the development of disease later in life, such as ASD (Diamanti-Kandarakis et al., 2009). This hypothesis is supported by studies that found significant associations between SNPs in estrogen receptors & enzymes involved in testosterone metabolism and the development of ASD. Furthermore, it has been shown that elevated levels of sex steroid hormones in boys diagnosed with autism when analyzing amniotic fluid (Koufaris & Sismani, 2015).

As seen above, the effects of EDCs on neurodevelopment can have dire consequences. However, controlling exposure to these chemicals has proven to be extremely difficult considering that, not only are there various sources for contamination, such as drinking contaminated water, breathing contaminated air, ingesting food, or contacting contaminated soil, but also because these bioaccumulate in the food chain, and some have a very prolonged life span, which allows them to migrate to areas where they were never produced, and sometimes are broken down into even more toxic compounds (Diamanti-Kandarakis et al., 2009). Other xenobiotics may not act as EDCs, but use other mechanisms to increase ASD risk.

1.3.2 METABOLISM

Metabolism is a key mechanism involved in degradation of xenobiotics, and it is a potential pathway thought to be disrupted in ASD: It is suggested that people who have defective xenobiotic metabolism will have increased sensitivity to certain environmental risk factors which can be easily processed by individuals with efficient metabolism. Xenobiotic enzymes are responsible for detoxifying and removing harmful chemicals from the body, primarily in the liver, but also in other parts of the body such as the brain. Polymorphisms affecting such enzymes have been associated with ASD (Koufaris & Sismani, 2015). These include polymorphisms in cytochrome p450 enzyme, responsible for metabolizing possibly toxic substances including pharmaceutical drugs, enzyme paraoxonase (*PON1*), involved in the removal of organophosphate pesticides from the body, and the metal-regulatory transcription factor 1 (*MTF1*), involved in metal homeostasis in response to heavy metals (Koufaris & Sismani, 2015).

The Cytochrome P*450* enzyme family has been especially studied due to its essential role in metabolism. In cells, Cytochrome P450 enzymes can be found in the mitochondria, where they are usually involved in the synthesis and metabolism of internal substances. They can also be found in the endoplasmic reticulum, where they generally metabolize external substances, mainly medication and environmental pollutants (Cytochrome p450, 2018). There are two phases in metabolization, and Cytochrome P450

enzymes are the main enzymes involved in phase I reactions. Phase I reactions include hydroxylation, reduction and oxidation, followed by phase II reactions such as glucuronidation, sulfation, acetylation and methylation. Another justification for the importance of these enzymes comes from the essential role they play in the metabolism of medications: cytochrome p450 enzymes are divided into families and subfamilies according to similarities in function, and cytochromes families 1 to 3 are responsible for the significant portion of 70% to 80% of all phase I dependent drug metabolisms, and also participate in the metabolism of a large number of xenobiotic chemicals (Wijnen et al., 2007). In general, 90% of metabolic activity fall on six enzymes: *CYP1A2*, *CYP3A*, *CYP2C9*, *CYP2C19*, *CYP2D6* and *CYP2E1*, showing the crucial role played by this family of enzymes in metabolism, and how imperative they are (Wijnen et al., 2007).

These family of enzymes is highly polymorphic, and each polymorphism will affect the efficiency of one's metabolization. This is also very important in the world of medicine, since these enzymes play such a vital role in the metabolization of pharmaceutical drugs. Depending on the polymorphism, and individual can be a ultra-extensive metabolizer (UEM), extensive metabolizer (EM), intermediate metabolizer (IM) and finally a poor metabolizer (PM), all according to the speed and efficiency of their metabolization (Wijnen et al., 2007) .A PM will have much slower enzymatic activity compared to a EM; since the enzymes in a PM work slower, this person will have more of the drug's active ingredient present in the body for longer period of time, whereas the EM will have less of the active ingredient and for a shorter period of time. Consequently, the polymorphism in Cytochrome P450 enzymes can affect medication dosage necessary. If the drug is slowly metabolized, less will be needed to achieve the desired effect, while more will be needed if it is metabolized fast (Cytochrome p450, 2018). This is crucial, as it may result in drug toxicity in an poor metabolizer if one is administered a high drug dosage or multiple drugs that are metabolized by the same cytochrome, and thus compete with each other (Wijnen et al., 2007).

The same mechanism applies to other xenobiotics such as environmental toxins. Therefore, it is believed that cytochrome p450 enzymes may be involved in the mechanisms which lead to an increased risk of ASD development: Poor metabolization of xenobiotics may lead to the accumulation of toxic substances in the body, which can therefore have negative effects and interfere with fetus' neurodevelopment. Thus, polymorphisms that affect the function of xenobiotic enzymes can make certain individuals more sensitive to environmental ASD risk factors (Koufaris & Sismani, 2015).

1.3.3 Physiological Barriers

The human body is protected by different permeability barriers such as the blood- brain barrier (BBB), the placental, intestinal and airway barriers that control our contact with external factors. The blood- brain barrier (BBB) plays an important role in CNS defense by limiting the entree of circulating solutes, macromolecules, and cells that could negatively impact neuronal activity (Fiorentino et al., 2016). The placenta, apart from the exchange nutrients and waste products between maternal and fetal circulatory system, it also protects the fetus from external chemicals (Gude, Roberts, Kalionis, & King, 2004). The intestine acts as a selectively permeable barrier permitting the absorption of nutrients, electrolytes and water, while defending the body from toxins and antigens (Groschwitz & Hogan, 2009). Finally, the airway barrier is responsible for clearing of inhaled pathogens, allergens and matter without inducing inflammation and maintain tissue homeostasis (Ganesan, Comstock, & Sajjan, 2013). It has been hypothesized that the malfunction of such barriers, can increase the risk of ASD development. Since chemicals have to pass by several of these obstacles before reaching the fetus' brain, they play a crucial role in protecting the fetus from the harmful substances that the mother may be exposed to: chemicals

have to cross several barriers such as the placental and blood barrier in order to gain access to the developing brain. While later in life, after birth, their protection will depend on skin, airway and intestinal barriers (Carter, 2016).

A study where genes previously associated with ASD were analyzed and related to barriers, found that a large number of these genes were placed and enriched in barriers and detoxification processes. Some of them were localized in the lateral ventricle, a cavity of the brain where xenobiotics and endogenous waste are eliminated from the cerebrospinal fluid to prevent accumulation in the brain. They were also highly enriched in blood-brain barrier, lung, skin, airway, respiratory cilia proteome datasets. Such results were also found in the liver, kidney and intestinal regions, which justify common ASD comorbidities such as gastrointestinal pathologies, as well as reports of impaired liver and kidney function (Carter, 2016). The significant enrichment of ASD genes in the intestine supports the hypothesis that inappropriate antigen trafficking through a damaged intestinal barrier, resulting in the passage of these antigens or immune-activated complexes through a permissive blood–brain barrier, can be part of the chain of events leading to neuroinflammation and thereby the development of disease. This increase of intestinal sensitivity to certain environmental factors explain why a controlled diet is recommended to patients with ASD. Many ASD patients experience gastrointestinal symptoms and dysfunctions which affect gut permeability, such as permeability to food antigens derived from partial digestion of wheat and cow's milk, make ASD sufferers more sensitive to what they eat (Fiorentino et al., 2016). Genes associated with ASD are also enriched in reproductive tissues such as the endometrium and the cervix, which play vital roles against infectious agents, but also against industrial pollutants that may have toxic effects on reproductive tissues. Therefore, if these barriers do not function properly, there is an increased chance of maternal infection during pregnancy, which several studies have linked to increased risk of ASD development in offspring. While ASD genes expressed in the brain are important for neuropathological effects, these expressed and enriched in the barrier functions, specially BBB, are also extremely important because their allocation is very relevant to the many pollutants related to ASD (Carter, 2016).

An example of ASD associated genes related to barrier functions are: transporters *SLC1A1* (glutamate) *SLC19A1* (folate) *SLC6A3* (dopamine) *SLC6A4* (serotonin) *SLC6A8* (creatine), and pumps *ATP1A1*, *ATP10A* which regulate chemical and ion entry at the cell membrane, placing the genes at an interface between environment and effect. Involved in placental function there is folate transporter (*SLC19A1*), as well as dihydrofolate reductase (*DHFR*) and 5,10-methylenetetrahydrofolate reductase (*MTHFR*), all involved in processing folate, an essential factor for embryonic development. As well as placentally localised genes like adenosine receptor (*ADORA2A*), Acetyl- cholinesterase (*ACHE*), and synaptic scaffold protein *SHANK2*. Dyslexia susceptibility 1 candidate 1 (*DYX1C1*), is involved in the regulation of respiratory cilia that sweep mucus, debris and toxic particles away from airways. And finally, genes such as glutathione peroxidase (*GPX1*) and transferase (*GSTM1*) and Paraoxonase 1(*PON1*) which are directly involved in detoxification processes such as that of heavy metals and organophosphate pesticides, respectively. All mechanisms previously associated with ASD development (Carter, 2016).

1.4 Prenatal, Perinatal & Neonatal Risks

Seeing as ASD has an early onset development , it comes as no surprise that a number of studies have been focused on maternal lifestyle, with attempts to correlate prenatal factors to ASD development. A study found that by every 10 year increase in maternal and paternal age, the increase of ASD risk in the

offspring is that of 18% and 21% respectively. In addition to this, it has also been hypothesized that the increase in ASD risk may be attributed to the accumulation of toxins, such as persistent organic pollutants (PCBs) and EDCs, in the body since it is probable that older parents lived in times prior to chemicals bans and experienced more years of accumulation (Kalkbrenner et al., 2014).

In terms of medication used during pregnancy, pharmaceutical drugs such as valproate has been associated with ASD in offspring on account on its effect on neurodevelopment (Kalkbrenner et al., 2014). It is also important to note that different studies have suggested the incidence of drug metabolism alteration during pregnancy. It is a well-known fact that hormone levels change during pregnancy: there is an increase of female hormone concentrations in the plasma, such as progesterone and estrogens. A study investigating the relationship between this increase in hormone level and the expression of drug-metabolizing enzymes, found that these hormones are capable of modulating expression and/or activity of certain drug-metabolizing enzymes (Jeong, 2010). On account of reported clinical changes during pregnancy, shown in table 1, researchers hypothesized that female hormones are possibly responsible for certain cytochrome p450 enzymes alterations during pregnancy. They found that estradiol up-regulates expression of *CYP2A6*, *CYP2B6*, *CYP3A4* and *UGT1A4*, but down-regulates *CYP1A2*, while progesterone up-regulates *UGT1A1* (Jeong, 2010).

Table 1. Altered drug metabolism during pregnancy: Elimination rates of drugs (in mother) metabolized by UGT1A4, UGT2B7, CYP2A6, CYP2C9, CYP2D6 and CYP3A4 are increased, whereas those of CYP1A2 and CYP2C19 substrate drugs are decreased. (adapted from Jeong, 2010)

CYP	Direction of activity change	Clinical evidence
CYP1A2	Decrease	Decreased apparent clearances or increased metabolic ratios of caffeine, theophylline, olazapine, and clozapine
CYP2A6	Increase	Increased clearance of nicotine
CYP2D6	Increase	Increased apparent clearances or decreased metabolic ratio of fluoxetine, citalopram, metoprolol, and dextromethorphan
CYP2C9	Increase	Increased apparent clearances of phenytoin and glyburide
CYP2C19	Decrease	Increased metabolic ratio of proguanil
CYP3A4	Increase	Increased apparent clearances of midazolam, nifenidine, and indinavir
UGT1A4	Increase	Increased apparent clearances of lamotrigine

Air pollution has also been associated with ASD: a study found elevated risk for ASD in adjusted analyses of the top quartile of exposure to chlorinated solvents, heavy metals, diesel particles and other individual compounds (Lyall, Schmidt, & Hertz-Picciotto, 2014). Although controlling exposure to such pollutants may be challenging, their interaction with a developing fetus may have dire consequences; metals are capable of crossing the placenta and blood-brain barrier, accumulate in developing brain and interact directly at the cellular level through a variety of mechanisms and causing reactive oxygen species (Kalkbrenner et al., 2014). Exposure may be due to residential proximity to freeway, traffic-related air pollution, high levels of modeled air pollution at home, etc. (Tordjman et al., 2014). Another study on air

pollution, with controlled sociodemographic factors, linked residence proximity to a freeway at the time of delivery with nearly a doubling in odds of having a child with ASD (Lyall et al., 2014). On the other hand, due to tobacco's renowned harmful effects, studies have explored its relationship to ASD but no significant association linked to maternal smoking during pregnancy has been found (Kalkbrenner et al., 2014).

Studies investigating prenatal factors are difficult to conduct and interpret since it is very unlikely that these factors are independent environmental risks. For example, studies liking maternal immigration to ASD are more likely to be due to lack of immunity against infectious agents in the country the mother gives birth in, and also due to maternal stress due to immigration itself that could be associated with social economic factors (Gardener, Spiegelman, & Buka, 2009). As for the relations found between gestational bleeding and ASD, this might be due to hypoxia, which in turn may be related to fetal distress, maternal bleeding, umbilical-cord complications, low Apgar, among other complications (Tordjman et al., 2014; Gardener et al., 2009). It is also challenging to study medication use due to the variety of medications used during pregnancy.

In terms of prenatal environmental factors that seem to decrease the risk of ASD development, studies with folic acid have produced the most consistent results. In a case-control study, consumption of prenatal vitamin supplements near the time of conception was associated with about 40% reduction in risk for ASD (Lyall et al., 2014). Yet another study found a lower incidence of ASD in children whose mothers received prenatal folic acid supplementation around the time of conception (64/61 042 or 0.10%) than in children whose mothers did not take folic acid (Tordjman et al., 2014). Therefore, it is suggested that maternal use of folic acid supplements during pregnancy could significantly reduce the risk of ASD in children as compared to those without folic acid supplementation (Wang, Li, Zhao, & Li, 2017). In addition to this, vitamin D has been shown to play a role in ASD onset. 25OHD is the metabolite used to measure levels of vitamin D, and studies shave show that lack or deficiency of 25OHD during gestation is associated with an increased risk of ASD development (Vinkhuyzen et al., 2017; Rebecca J. Schmidt et al., 2015; Cieślińska et al., 2017; Chen, Xin, Wei, Zhang, & Xiao, 2016). A study has found that, compared with individuals with sufficient concentrations of 25OHD at mid-gestation, those that were deficient had a more than twofold increased risk of ASD (Vinkhuyzen et al., 2017). Yet, another study also found negative association between risk of ASD and maternal 25OHD serum, but on the first trimester: they found that mothers in autistic children had significantly lower serum levels of 25OHD than mothers of typically-developing children (Chen et al., 2016). Thus, studies suggest that maternal levels of vitamin D during gestation can affect fetus' neurodevelopment.

It appears that no specific factor has been consistently validated as an independent environmental risk factor for ASD (Tordjman et al., 2014). Therefore, future environmental studies need to include combinations instead of trying to single out risk factors.

1.4.1 Breastfeeding and EDCs

Special attention has been attributed to breastfeeding since this is the main source of nutrition of a newborn, and also on the account that colostrum has been recognized as a font of EDC contamination since this is one of the most effective methods of excreting lipophilic chemicals (Kalkbrenner et al., 2014). A study that measured phenols in colostrum, found elevated levels of BPA being transmitted from mother to infant. This is worrying due to the fact that, unlike adults, newborns do not have fully developed detoxification enzymes, and in addition to this, it is believed that they have a higher body burden considering their low body weight and high susceptibility. Thanks to a questionnaire, the study also linked

this increase in colostrum BPA with maternal consumption of dairy products. This is believe to be a result of the packaging and storage processes which put the food in direct contact with these materials, and leads to contamination (Yi et al., 2013). These results are important to ASD studies, considering that EDC has been linked to neurodevelopmental damage, and therefore increase of ASD risk.

1.5 ELEAT

The Early Life Exposure Assessment Tool (ELEAT), originally developed in California by researchers of University of California Davis, is a questionnaire that aims to assess environmental exposures that have been associated with ASD development. It allows for an indirect approach given that it uses measurements of exposures in specific microenvironments (e.g. home) and human activity pattern (e.g. maternal lifestyle) data to predict levels of fetal/infant exposure. For example, to measure vitamin D synthesis, the mothers were asked about the number of hours spent outside during sunny periods while pregnant, instead of a direct measurement of vitamin D levels in plasma. Indirect exposures, such as this one, are used frequently on account that they require less resources than the assessment of direct exposures.

The long-term goal of this questionnaire is to assess the role of environmental exposures during early life windows of susceptibility relevant to ASD. However, as previously stated in Environmental Factors and Autism Spectrum Disorder section, is has been suggested that gene-environment interactions play an important role in ASD onset. Thus, to our understanding, in order to complement the ELEAT, there is the need to also address genetic factors that may modulate our organisms' response to external factors.

It is important to mention that, although different countries have been working with this tool, to our knowledge no results have been published to date.

2. GOALS AND STRATEGY

The pilot study here presented is part of a broader project and its main goal is to contribute to the better understanding of gene-environment interactions in ASD. For this, from a sample of children with the disorder, we aimed to collect information regarding early environmental exposure, through the application of the ELEAT questionnaire, in addition to analyzing genetic data. Thus, by joining these two complementary approaches, the objectives of this pilot study are to:

i. Define the type of data that can be obtained from the ELEAT questionnaire and how such data can be analyzed and interpreted;
ii. Define how results regarding environmental exposure obtained through the ELEAT can be related to genetic information collected from the same subjects;
iii. Identify links between environmental exposures and genetic variants that can be further explored in gene-environment studies in ASD.

In order to reach these goals, the following strategy (figure 2) was applied:

- Contact parents of children diagnosed in ASD and invite them to participate in the project by filling out the ELEAT.
- Collect biological samples from the affected children, for DNA extraction.
- Analyze ELEAT questions in order to know what environmental factors are being investigated in each question.
- Select genes that interact with the environmental factors investigated in ELEAT.
- Select polymorphisms that may interfere with gene function, and consequently, disrupt organisms' response to exogenous factors.
- Investigate if these genetic variants were present in the sample of probands.
- Investigate whether probands carrying these variants were exposed to the specific environmental factors they interact with.

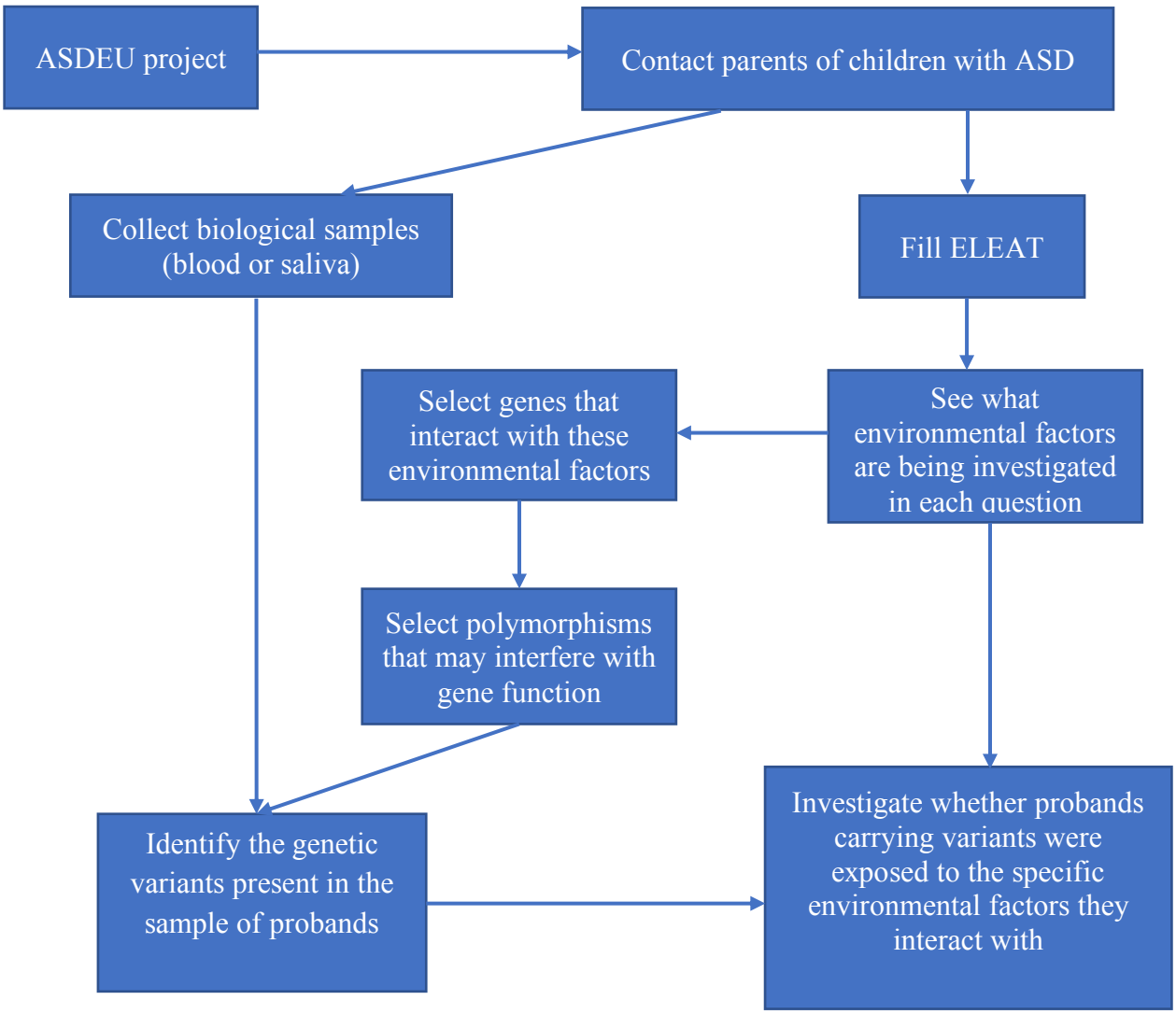


Figure 2. Scheme of strategy used to achieve the study's objectives

3. METHODOLOGY

3.1 PARTICIPANTS

3.1.1 ASDEU PROJECT

Participants for this study were recruited from the Autism Spectrum Disorder in the European Union (ASDEU) project. This aimed to investigate the prevalence of autism in 12 countries in the European Union, including Portugal, which was finalized in 2017. For this project, teachers of primary schools located in the Center Region of Portugal, were invited to fill in a questionnaire where they should nominate children in their class presenting characteristic ASD behaviors, such as: social awkwardness, intense interest in just a few topics or activities, reduced flexibility, tendency to insist on certain rules and routines, among others. The Central Region was the area chosen for this project considering that it includes 31.3 % of mainland Portugal and 23.7% of its population, and also on account that the Pediatric Hospital of Coimbra, which works closely with our department in INSA (National Health Institute Doutor Ricardo Jorge), is the reference pediatric hospital in that region and has clinical registers of a large percentage of ASD children. Moreover, the selected region includes both urban and rural neighborhoods, being an effective proxy for the whole Portuguese population and the whole array of possible environmental exposures.

As part of the ASDEU project, after the children were nominated by the teachers and the parents signed the informed consent, children were evaluated in the Hospital of Coimbra for the ascertainment of ASD, by an experienced clinical team. The diagnosis was carried out by applying ADOS, ADIR, Vineland, WISC III or Griffinths, thus, resulting in 54 diagnosed children. Simultaneously, biological samples were collected. However, some families had already been recruited for previous projects and, consequently, we already had their biological samples, which were processed by INSA and stored with anonymized codes.

It was from this list of families that parents were contacted and invited to be part of the ELEAT project. For individuals who did not have biological samples, buccal swab samples were collected and were also anonymized.

3.2 MATERIALS

3.2.1 ELEAT

The ELEAT's objective is to understand how environmental risk factors can influence ASD development.

This tool is divided in 10 modules: Demographic Information, Maternal Conditions/Medical Interventions, Breastfeeding and Child Diet, Maternal Diet, Supplements, Lifestyle, Home and Environment, Environment, and Occupation and Exposures, and Instrument Evaluation. This questionnaire is constituted by 259 items (some may be skipped with gateway questions), corresponding to 80 pages, which intend to assess 3 different periods: it examines maternal exposure to exogenous factors from 3 months before conception to the first year of the child's life. Therefore, covering crucial phases of early neurodevelopment, with each module analyzing different sources of exposure.

The questionnaire, which is to be filled by the mothers of the children with ASD, was translated into Portuguese from the UK version due to the greater similarities in lifestyle compared to the US. For example,

the American ELEAT included questions regarding fish types that were not available in Portugal, as well as housing typologies.

In addition to the paper version, an online version was created in the RedCAP survey, a secure web application for building and managing online surveys and databases. Here the mothers were able to access the questionnaire through a link, and were given the option to save their answers and proceed filling it in later. This became the main filling method not only because it was more organized, but mainly because, seeing as the questionnaire is extensive, the fact that the mothers could save their answers and return to it later made it easier and more appealing due to busy lifestyles. However, the mothers had always the chance to fill out the questionnaire in paper version.

After translation, the ELEAT was piloted in a group of mothers of typically-developing and ASD children, ranging from 1 to 8 years. All mothers had a university degree, and were between 35 and 44 years old.

This was done with the objective of obtaining feedback from the mothers regarding the questionnaire, such as clarity, length and relevance of the questions. This was done by analyzing the last module of the tool, which consists of questions were the mothers can rate the instrument.

3.3 PROCEDURE

3.3.1 CONTACTING PARENTS

For this project, we contacted a total of 54 parents (with children diagnosed with ASD) through the phone, and after a brief explanation about the project and its goals, they were invited to participate by filling the ELEAT. The parents who agreed provided us with their email, to which we sent a link for the ELEAT's online version, along with an explanation about the steps to take. After the initiating the filling of the tool, the website attributed an access code which allowed mothers to return to the application and continue the filling out process. After a period of time of inactivity, the parents who did not fill the questionnaire were contacted again as a reminder: some were contacted through the phone, while the ones who had started filling the questionnaire, but did not conclude it, were sent an email with their access code and offered help to clarify any questions. This process had to be repeated several times, and for those who had incomplete questionnaires we offered phone assistance: to fill the questionnaire with the parents by asking them questions through the phone, and clarifying any doubts.

As three mothers did not have email, they were unable to access the ELEAT online. Thus, we offered personal assistance to them, by traveling to their homes in the center region of Portugal and help them fill out the questionnaire. Such travels were necessary, not only because we could not send the link of the online version, but also to ensure that there were no doubts regarding the questions.

3.3.2 COLLECTION OF SAMPLES

Biological samples were only collected from the children whose mothers filled out the questionnaire but did not have samples collected at the moment of diagnosis. This was done using swab kits from iSWAB^{tm}. A total of 12 parents were contacted though the phone and asked if they were willing to provide a biological sample, to which all of them agreed. They were given two options: we could travel to their houses and collect the sample ourselves, or we could send them the swab kit by mail with clear instructions on how to use them, which they could then send back to INSA in a prepaid package. All parents preferred to collect the sample themselves and send it back to us.

After a certain period of time, the parents who had not returned the samples were sent an email to know if the kit had not arrived, or if they were having trouble with the process, to which we offered our assistance.

3.3.3 Selection of ELEAT questions

As the ELEAT questionnaire is very extensive and, as we intended to focus our research, we selected a total of 156 of questions (without counting the sub-questions) to further analyze. We chose questions that allowed us to more directly assess probable exposures to environmental toxins relevant to ASD, which have been well reported in literature. Additionally, we also selected questions that evaluate exposure to other environmental factors which are hypothesized to be relevant to ASD, such as the intake of certain supplements like folic acid, and vitamin D during the period covered by the ELEAT. As addressed ahead, it was also important that the organism's response to the evaluated factors could be clearly related to specific genes. In summary, we selected questions where:

- The rationale and environmental factor being analyzed was sufficiently clear;
- Most of the environmental factors have been associated with ASD risk;
- Specific genes have been related to these environmental factors.

3.3.4 Presentation of ELEAT results

Out of the 54 contacted parents, a total of 20 mothers filled the questionnaire. Seeing as some mothers left questions unanswered, percentages were always calculated based on the total number of answers to each selected question. The questionnaire had a number of sub-questions which were indicated only for mothers who answered "yes" in the main question. E.g.: Module B, question 4: "Did you feed your child with infant formula?". Only the mothers who answered positively were to move on to question 4.c. "What type of formula did you use?", while the other mothers would move to question 5. For this reason, the results presented in the sub-questions are out of the number of mother who responded affirmatively in the main question.

3.3.5 Selection of genes and polymorphisms:

The choice of genes to be tested was based on available knowledge from literature

i. Genes involved in the activity of environmental factors

After compiling a list of the environmental factors being assessed in each of the selected questions, we searched for genes involved in the activity or regulation of barriers' permeability to such factors. For that, we used the Comparative Toxicogenomics Database (CTD), a manually curated platform that provides information about interactions between chemicals and gene products, and their relationships to diseases (Davis et al., 2017). The CTD was used in the following way: after obtaining the MeSH ID (a unique identifier assigned to each chemical by the Medical Subjects Headings (https://www.nlm.nih.gov/mesh/) for each factor, each ID was inserted individually in CTD, after which a txt file could be downloaded. This file included the genes this factor interacts with, which were repeated according to the number of published references supporting each interaction. Due to this repetition, we were able to assign higher support to genes more frequently referenced . As a result, each factor had a list of genes they interact with, and these were ordered from higher to lower number of references. The genes that were chosen were those with higher support..

From this list of selected genes, we chose to analyze functional polymorphisms; these are polymorphisms that disturb gene function by affecting the functionality of the produced protein. We selected those whose effect on protein has been well studied. In addition to this, we showed preference to

those that have higher population frequencies in order to increase the chances of finding them in a small sample.

ii. Genes involved specifically in the activity of pharmaceutical drugs

In order to select which genes involved in medications effects we should analyze, we started by exploring ELEAT's Maternal conditions/Medical interventions Module, where each medication taken by the mothers was annotated. To find the active ingredients for each of these medications, we resorted to Infomed (http://app7.infarmed.pt/infomed/), the medication database of the website Infarmed, which contains information on human medication, namely the name of the medicinal products, active substances (INN / generic name), dosage, pharmaceutical form, among others.

Next, the names of the active ingredients were inserted in the database PharmGKB: a pharmacogenomics knowledge resource with information about the impact of genetic variation on drug response (Altman, 2007). To collect the data we needed, we used the *Clinical Annotations* section which gave us information about variant-drug pairs, based on variant annotations in the database. Here we were able to identify the genes these medications interacted with, the specific polymorphisms that affect their function, as well as the type of effect they have: such as toxicity, efficacy, dosage, among others. Additionally, the level of evidence for each polymorphism was also listed.

With this information, a table was created with medications' name, their active ingredient, and the genes and specific polymorphisms they interacted with. From here, we excluded HLA (Human Leukocyte Antigens) genes due to their complexity, and polymorphisms that affected factors other than toxicity and efficacy (see attachment S1). This is because we were investigating toxic effects that medication and other environmental factors may have on the fetus if this carries a variation that makes it particularly vulnerable to that factor. Efficacy is included because, if a variation affects the efficacy of the metabolization of a medication, this can accumulate in the body and therefore become toxic.

To clarify the genotype (the alternative allele) of each polymorphism, as well as the frequency in European (non-Finnish) and African populations, we used PharmGKB, GnomAD, a database that gathers exome and genome sequencing data from a wide variety of large-scale sequencing projects (Lek et al., 2016), and ClinVar, a database belonging to NCBI which aggregates information about genomic variation and its relationship to human health (Landrum et al., 2014).

Selected genes and genetic variants are displayed in table 2.

3.4 LABORATORY PROCEDURES

3.4.1 DNA EXTRACTION

The DNA extraction of all the biological samples used was done at INSA, in the Health Promotion Department (DPS).

From blood:

The protocol used to extract DNA from blood was based on the "salting out" method described by D.K. Lahiri *et al* (1991). Since this is done from white blood cells, one of the first steps is to eliminate the erythrocytes followed by the deproteination, in order to be left only with white blood cells, which are nucleated and where genomic DNA can be extracted from human blood. For more details, please refer to attachment S2 for the full protocol.

From buccal swab sample:

The protocol used for buccal swab samples was adapted (inhouse method) from "salting out" method described by D.K. Lahiri *et al*, skipping the elimination of erythrocytes steps, and using buccal mucosa instead of white blood cells. This protocol was applied on swab samples collected with iSWAB^{tm} - DNA from mawi DNA Technologies, LCC.

For more details, refer to attachment S3 for the full protocol.

Table 2: Genes, polymorphisms, variant annotations and analysis techniques used

PharmGKB		PharmGKB & GnomAD		Technique	Reference
Gene	Polymorphism	Genotype	Annotation		
ABCB1	rs1045642	G > A	Synonymous	PCR and Digestion with MboI enzyme (PCR-RFLP)	Jamroziak et al., 2002
ACHE	rs2571598	C > T	Intronic	Sanger Sequencing	
AHR	rs4410790	T > C	*unannotated*	Sanger Sequencing	
CYP1A1	rs2606345	C > A	Intronic	Sanger Sequencing	Zhao et al., 2017
CYP2C19	CYP2C19*2	G > A	Synonymous, creating an aberrant splice site	Sanger Sequencing	
CYP2D6	CYP2D6*4	C > T	Splice-acceptor region	Sanger Sequencing	
	CYP2D6*6	del. T	Frameshift	Sanger Sequencing	
CYP2R1	rs10741657	A > G	Promoter Region	PCR and Digestion with MnlI enzyme (PCR-RFLP)	Ramos-Lopez et al., 2007
CYP3A4	rs2740574	C > T	5-prime promoter region	Sanger Sequencing	
GSTM1	GSTM1*0	homozygous GSTM1*0	GSTM1-null	PCR (based on the length of PCR product)	
HBB (internal control)	NA	NA	NA		Kendi Takeda et al., 2011
MTHFR	rs1801133	C > T	Missense	PCR and Digestion with HinfI enzyme (PCR-RFLP)	Chandra et al., 2009
OXTR	rs2268498	T > C	promoter flanking region of OXTR	Sanger Sequencing	
PLCG1	rs2228246	A > G	Missense	Sanger Sequencing	
PON1	rs854560	A > T	Missense	Sanger Sequencing	
	rs662	T > C	Missense	Sanger Sequencing	
SLC19A1	rs1051266	A > G	Missense	PCR and Digestion with CfoI enzyme (PCR-RFLP)	Coppedè et al., 2014
TNFRSF11A	rs1805034	C > T	Missense	Sanger Sequencing	
UGT1A	rs1042640	G > C	3 UTR	Sanger Sequencing	
*UGT1A gene cluster**	rs10929303	T > C	3'UTR	Sanger Sequencing	Court et al., 2013
	rs8330	G > C	3'UTR	Sanger Sequencing	Court et al., 2013
UGT2B15	rs1902023	A > C	Missense	Sanger Sequencing	Alkharfy et al., 2017
VDR	rs731236	T > C	Synonymous	PCR and Digestion with TaqI enzyme (PCR-RFLP)	Cieslinska et al., 2017

**UGT1A gene cluster (UGT1A1, UGT1A10, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9). In gray are polymorphisms not found in PharmGKB.*

3.4.2 Primers Design

By inserting the genomic coordinates of each polymorphism into the UCSC genome Browser (hg38/Human) website (https://genome.ucsc.edu/cgi-bin/hgc?g=getDna&i=hg38), we were able to find the genomic region that contains each polymorphism. Primer3 software was used to design forward and reverse primers. and to gather information regarding the melting temperature (ºC), and guanine-cytosine content (gc%).. In order to verify that there were no single nucleotide polymorphisms (SNPs) in each primer, we used the website SNP Check3. This informed us of any SNPs in the primer region and where exactly it was located so that the primer could be redesigned. Lastly, to confirm the specificity of the primers we used the BLAST tool from NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). This gave us information on the whether the primers would bind specifically to the location we intended, with a 100% specificity. Primers used are displayed in table 3.

Table 3. Forward and reverse primer sequences for each polymorphism being studied

Gene	RS	PrimerForward	PrimerReverse
ABCB1	rs1045642	TTGATGGCAAAGAAATAAAGC	CTTACATTAGGCAGTGACTCG
ACHE	rs2571598	AGTGCAGTGGTGCCATCATA	GGGTCTTGCAGAGACAGAGG
AHR	rs4410790	TGACTCCTATTGTTGTGAATAAAAGC	CGGAAAAGCATTCTGAAGGA
CYP1A1	rs2606345	GGGAGATGGATGGTTCCTACCAC	CCTCCTAAGGGTGGCTTGTCAGT
CYP2C19	rs4244285 (CYP2C19*2)	CAACCAGAGCTTGGCATATTG	TCACAAATACGCAAGCAGTCA
CYP2D6	rs3892097 (CYP2D6*4)	TCCTGAGCTAGGTCCAGCAG	ATAGGGTTGGAGTGGGTGGT
	rs5030655 (CYP2D6*6)	TCCTGAGCTAGGTCCAGCAG	ATAGGGTTGGAGTGGGTGGT
CYP2R1	rs10741657	GGGAAGAGCAATGACATGGA	GCCCTGGAAGACTCATTTTG
CYP3A4	rs2740574	GGAAGAGGCTTCTCCACCTT	CTGGGTTTGGAAGGATGTGT
GSTM1	*GSTM1*0*	TTTGCTCCGTTAGGATCTGG	GAGGGTACTGCAGGAGACCA
	GSTM1 +/+	ACAGTGAGTGCCTGGTCTC	TCAGGGCTGTAGCAGACTCT
HBB (internal control)		CAACTTCATCCACGTTCACC	GAAGAGCCAAGGACAGGTAC
MTHFR	rs1801133	TGAAGGAGAAGGTGTCTGCGGGA	AGGACGGTGCGGTGAGAGTG
OXTR	rs2268498	AGGTCCTGTCCCTCTGGTTT	TAGGCTGTCTCACGGGCTAC
PLCG1	rs2228246	GTGTCCCTTCCTGAGTTCCA	GTCCTGGTCACCCCTACTCA
PON1	rs662	CAAATCCTTCTGCCACCACT	AAGGATTGTATCGGCAGGAC
	rs854560	TGGTTCAATGTAGACCGAAGAA	TGGATCCACATCCTGCAATA
SLC19A1	rs1051266	AGCGTCACCTTCGTCCC	TCCCGCGTGAAGTTCTTG
TNFRSF11A	rs1805034	CCAAAGCACTGAACCACCTT	CCCCCAATCCAGTGTAGAAA
UGT1A	rs1042640	GCATAAATTAATCAGCCCCAGAGTGC	CACCACCCACCAATTTCATAGCATC
UGT1A cluster	rs10929303	GCATAAATTAATCAGCCCCAGAGTGC	CACCACCCACCAATTTCATAGCATC
	rs8330	GCATAAATTAATCAGCCCCAGAGTGC	CACCACCCACCAATTTCATAGCATC
UGT2B15	rs1902023	GAGCTTGTTCAGAGGGGTCA	CAAAACTGCATCTTTACAGAGCTT
VDR	rs731236	GGATCCTAAATGCACGGAGA	AGGAAAGGGGTTAGGTTGGA

3.4.3. Polymerase Chain Reaction (PCR)

For fragment amplification PCR we followed the protocol for Applied Biosystems AmpliTaq DNA Polymerase, and a mix was made with buffer, $MgCl_2$, dNTPs, DNA Polymerase and H_2O. The volume of the mix was calculated so that each tube would receive 22ul. After this, 1ul of each primer specific to the polymorphism being analyzed was added to each tube. Finally, each tube received a 0,5ul of DNA, to make a final volume of 25ul.

The reactions were run under the following thermocycling conditions: 94ºC for 1 min; 30 cycles of 94 ºC for 30 sec, 57-62 ºC for 30 sec (depending on the melting temperatures of each pair of primers) and 72 ºC for 1 min, followed by 72ºC for 5 min and 4 ºC as resting temperature. For this, T3000 a programmable thermocycler from Biometra was used.

The PCR products were ran in a 1.8% electrophoresis gel stained with 1.6ul of SYBR Safe DNA gel stain (Invitrogen). 5ul of each PCR product were mixed with 5ul of deposition solution and inserted into the wells where they were then run in 90V for 40 minutes. Afterwards, the PCR products were purified by mixing 2ul ExoProStar x 5ul PCR product, and ran on the thermocycle under the following conditions: 37 ºC for 15 min, 80 ºC for 15 min and lastly 4 ºC as resting temperature.

3.4.4 Sanger sequencing

In order to prepare the samples sequencing, in each tube containing 1ul of PCR product, we added: 0.5ul of sequencing buffer, 2ul of forward primer, 0.5 of BigDye terminator reaction mix, and 6ul of H_2O. These were then run in the thermocycle with 96 ºC for 1 min; 25cycles of 96 ºC for 10 s, 58 ºC for 5 sec, 55 ºC for 4 sec, and lastly 4 ºC as resting temperature. The tubes were then sent to a specialized group in the department of Human Genetics at INSA to be sequenced by capillary electrophoresis. Chromatogram analyzes were done with Chromas software (Technelysium Pty Ltd).

3.4.5. Restriction fragment length polymorphism (RFLP)

As shown in table 2, some of the selected polymorphisms were detected through RFLP. This is on account that for these polymorphisms this technique is well established. For all these variants PCR amplification was performed in a thermal cycler (T3000 thermocycler from Biometra) using the primers listed in table 3. Each PCR reaction contained 25ul of total volume (2.5ul PRC Buffer, 1.5ul $MgCl_2$, 0.5ul dNTP mix, 1ul forward and 1ul of reverse primer, 0.3 of AmpliTaq DNA polymerase, 0.5ul of genomic DNA and 17.7ul of H_20). 5ul of the PCR products were ran in in a 1.8% electrophoresis gel stained with 1.6ul of SYBR safe dye. After checking for correct amplification, each mixture was subjected to 4 hour digestion at 37 ºC with the restriction enzymes referred in table 4, using CutSmart Buffer and BSA (Bovine Serum Albumin). Finally, genotypes were assessed by running the digested products, shown in table 4, in a 3% electrophoresis gel.

3.4.6. PCR (detection based on the length of PCR product)

This protocol was used specifically for detecting *GSTM1* genotype seeing as it is the only variant that is not a point mutation: the polymorphism corresponds to the deletion of the entire gene. Fragment amplification was done as described in Sanger Sequencing section, followed by electrophoresis in 1.8% agarose gel, which displayed different patterns depending on the genotype.

For each of the 14 subjects two reactions were done: the first with primers specific for the upstream and downstream flanking regions of *GSTM1* and the second with primers specific for a region inside *GSTM1*. In the gel (attachment S8) we used two wells per individual, with the first one corresponding to the deletion and the second one to the presence of the gene. Individuals homozygous for the deletion are expected to display a single DNA band of 433bp (in the first well), while individuals with no deletion are expected to display a band of 379bp (in the second well). Heterozygous individuals are expected to display both of these bands.

As positive control, we used a pair of primers specific for a housekeeping gene. This was to ensure that amplification would happen for all subjects. The chosen gene was *HBB* (Hemoglobin Subunit Beta).

Table 4 Fragments obtained after RFLP digestion for polymorphisms being analyzed by this technique

Gene	rs	Restriction Enzymes	PCR product size (bp)	RFLP digestion fragments
ABCB1	rs1045642	MboI	206	AA genotype: 206; GG genotype: 130+76; GA genotype: 130 + 76 / 206
CYP2R1	rs10741657	MnlI	288	GG genotype: 300; AA genotype: 2048+52; AG genoype: 248 + 52 / 300
MTHFR	rs1801133	HinfI	198	TT genotype: 53 + 125; CC genotype: 198; CT genotype: 198 / 53+ 125
SLC19A1	rs1051266	CfoI	230	GG genotype: 125+105; AA genotype: 230; AG genoype; 230 / 125+105
VDR	rs731236	TaqI	630	CC genotype: 630; TT genotype: 425+205; TC genotype: 425+205 / 630

4. RESULTS

4.1 ELEAT QUESTIONS & GENES

After following criteria for the selection of ELEAT questions, and the respective genes that interact with the environmental factors being analyzed in each question, we obtained table 5. A total of 18 genes, comprehending 22 polymorphisms, were chosen to analyze. As it can be seen in the referred table, the selected questions assess exposure to different xenobiotics, from medications to environmental toxins (e.g. BPA, PBDEs, PCBs, heavy metals and pesticides). There is significative overlap between the factors processed by each of the selected genes, which was expected since most of those genes encode for key enzymes involved in biotransformation pathways. However, some genes are more specific to particular factors, such has *MTHFR* and *SLC19A1* to folic acid, *CYP2R1* and *VDR* to vitamin D and *OXTR* to oxytocin. Additionally, as previously referred, we prioritized the choice of exonic vs intronic polymorphisms, which can be seen by their higher frequency on the table. All of the selected polymorphisms are point mutations that change or delete a single nucleotide, apart from GSTM1*0 variant, which refers to the whole deletion of the gene. Most of the genes were selected due to their obvious role in the activity of environmental toxins. However, others (e.g. *OXTR*, *PLCG1* and *TNFRSF11A*) were chosen due to the effect of certain polymorphisms in the effect of medications taken by the mothers' who answered the ELEAT questionnaire.

In table 6 is the functions of the selected genes and how these can be affected by the polymorphisms being studied. The genes have a wide variety of functions: some are receptors (e.g. *AHR*, *OXTR, TNFRSF11A* and *VDR*), transporters (e.g. *ABCB1* and *SLC19A1)* Cytochrome P450 enzymes (e.g. *CYP2C19*, *CYP2D6, CYP2R1*) and other metabolizing-enzymes (i.e. *GSTM1*, *MTHFR*, *PON1*, UGT1A gene cluster, *UGT2B15).* Importantly, all of these genes have known roles in metabolization or detoxification of environmental factors or medications. As for the polymorphisms' effect, most of them result in a reduced function of the protein, while some may lead to an increased activity. Such activity alterations may result in an improper response to external cues.

Table 5. Results obtained after following criteria for the selection of ELEAT questions and genes that interact with the environmental factors being analyzed in each question. Module B: Breastfeeding & Child diet, Module M: Maternal Conditions/ Maternal Interventions, Module D: Maternal Diet, Module S: Supplements, Module L: Lifestyle, Module H: Home & Environment, Module E: Environment, Module O: Occupation & Exposure

Questions	Factor Being Studied	Genes
Module H: Q.20, 22, 23 **Module E:** Q. 1, 3, 4, 5, 24-32, **Module O:** Q.15-22, 24, 25, 27	Multiple xenobiotics (i.e. PAHs, PBDEs, PCBs, and phthalates)	*CYP1A1*
Module S: Q. 1, 2, 5	Folic Acid	*MTHFR*
		SLC19A1
Module L: Q. 3, **Module D:** Q.5, 25, 26 &29	Vitamin D; 25(OH)D	*CYP2R1*
		VDR
Module H: Q.13, 17, 18 **Module L:** Q. 10, 17	PAHs (benzo(a)pyrene)	*AHR*
Module H: Q.20, 22, 23 **Module E:** Q. 1, 3, 4, 5, 24-32, **Module O:** Q.15-22, 24, 25, 27	Multiple xenobiotics such as phthalates, PBDEs, PCBs etc.	UGT1A gene cluster
Module B: Q. 5, **Module D**: Q.3	Bisphenol A	*UGT2B15*
Module H: Q.20, 22, 23 **Module E:** Q. 1, 3, 4, 5, 24-32, **Module O:** Q.15-22, 24, 25, 27	Multiple xenobiotics such as phthalates, PBDEs, PCBs etc.	*GSTM1*
Module E: Q: 6-10 **Module H:** Q. 11, 21, **Module O:** Q.23	Organophosphate pesticides (i.e. chlorpyrifos)	*PON1*
		ACHE
Module D: Q.26, **Module H:** Q. 11&12, **Module E:** Q.33, **Module L**: Q. 10, 17	Multiple xenobiotcis including heavy metals and pharmaceutical drugs	*ABCB1*
Module M: Q.27	Oxytocin	*OXTR*
Module M: Q. 28, 45-58, 61- 68, 71 -82	Multiple xenobiotics but mainly pharmaceutical drugs	*CYP3A4*
		CYP2C19
		CYP2D6
	Pharmaceutical drugs but specifically Acetaminophen	*UGT1A*
		PLCG1
		TNFRSF11A

Table 6. Function of the selected genes and effect of each of polymorphisms being studied

Gene	Gene Fucntion	rs	Change	Polymorphism Effect	Reference
ABCB1	Encodes for p-glycoprotein, an efflux transporter that limits the movement of multiple xenobiotics, such as heavy metals and pharmaceutical drugs, across the blood brain barrier and placenta	rs1045642	G>A	Allele A is associated with decreased expression of p-glycoprotein in some tissues, including the placenta, and therefore with diminished function	Brambila-Tapia 2003, Mollgard et al 2007, Miller et al 2009, Walker et al 2017
ACHE	Is an enzyme that hydrolyzes the neurotransmitter acetylcholine. This enzyme is also the primary target of inhibition by organophosphate pesticides.	rs2571598	C>T	Allele T confers reduced acetylcholinesterase activity, which leads to reduced acetylcholine breakdown and thus less neurotransmission activity.	Reale et al 2018, Iossifov et al 2015, Lionetto et al 2013
AHR	Receptor for many toxins. Upon binding of the toxin to *AHR*, there is an induction of xenobiotic-metabolizing enzymes such as cytochrome P450	rs4410790	T>C	Allele C increases metabolism by inducing a higher expression of *CYP*s by *AHR*	Cornelis et al 2014, Moorthy et al 2015
CYP1A1	Enzyme involved in phase I metabolism of multiple xenobiotics, including PAHs, phthalates; many pharmaceutical drugs	rs2606345	C>A	Allele A confers higher enzymatic activity to *CYP1A1*	Moorthy et al 2015, Sulem et al 2011
CYP2C19	One of the most important CYP450 drug metabolizing enzyme in phase I expressed mainly in the liver	CYP2C19*2	G>A	Major loss-of-function SNP resulting in reduced enzymatic activity of *CYP2C19*. Homozygous genotype (*CYP2C19*2 /CYP2C19*2*) are poor metabolizers, while *CYP2C19*1/ CYP2C19*2* are intermediate metabolizers	Scott et al 2012
CYP2D6	Major CYP450 drug metabolizing enzyme in phase I expressed in liver, gut, and brain	CYP2D6*4	C>T	Allele T causes a splicing defect that results in a low-activity protein. This is the main cause of CYP2D6 poor metabolizers in Caucasians	Zanger et al 2004
		CYP2D6*6	deletion of T	Deletion of T is a frameshift mutation that results in a truncated, low-activity version of *CYP2D6*	
CYP2R1	Enzyme that catalyzes the conversion of vitamin D to 25(OH)D, the main circulating vitamin D metabolite and the main ligand to *VDR*	rs10741657	A>G	Allele G confers low activity to *CYP2R1*, and therefore leads to lower levels of 25(OH)D	Schmidt et al 2015
CYP3A4	One of the predominant xenobiotics-metabolizing enzyme expressed in human liver with substrates including a large amount of pharmaceutical drugs, in addition to many xenobiotics	rs2740574	T>C	This polymorphism may lead to increased transcription of *CYP3A4,* which may result in higher levels of the enzyme	Basheer & Kerem 2015
GSTM1	Encodes for a glutathione S-transferase enzyme, which is involved in the detoxification of electrophilic compounds, including environmental toxins, by conjugation with glutathione in phase II metabolism	GSTM1*0	homogygous GSTM1*0	Deteletion results in reduced or no *GSTM1* enzymatic activity, depending on the number of alleles deleted	James et al 2006, Buyske et al 2006
MTHFR	Reductase involved in folic acid metabolism: catalyzes the conversion of 5,10-MTHFR to 5-methyl-folate, the circulating form of folic acid, which is a substrate necessary for the remethylation of homocysteine to methionine	rs1801133	C>T	Allele T results in reduced circulating folic acid due to lower *MTHFR* activity. Compared to the most common genotype (C;C): Homozygous (T;T) individuals have ~30% of enzyme activity, Heterozygotes (C;T) have ~65% enzyme activity	Liu et al 2011, James et al 2010, Schmidt et al 2012

Table 6(continued) Function of the selected genes and effect of each of polymorphisms being studied

Gene	Gene Fucntion	rs	Change	Polymorphism Effect	Reference
OXTR	Encodes for a G-protein that functions as oxytocin receptor	rs2268498	T>C	The C allele is associated with higher mRNA expression of *OXT* receptor, which may reflect a reduced sensitivity to oxytocin	Montag et al
PLCG1	Involved in mast cell activation, which is important for neuroimmune activation, releasing histamine and other substances during inflammatory and allergic reactions. It also plays an important role in defense against pathogens, and blood–brain barrier function	rs2228246	A>G	Allele G has been astudied in reation to risk of Angioedema (a form of severe swelling beneath the skin's surface) when treated with acetaminophen as compared to genotype AA. However, results reagrding the association are unclear.	Ayuso et al 2015, Polyzoidis et al 2015
PON1	Produces an enzyme that is involved in the inactivation by hydrolysis of organophosphate pesticides	rs854560	A>T	Allele T & C result in reduced activity of paraoxonase enzymes, therefore less hydrolysis of organophosphates	D'Amelio et al 2005
		rs662	T>C		
SLC19A1	Folic acid transporter, involved in intracellular levels of folic acid	rs1051266	A>G	Allele G confers low activity to *SLC19A1*	Liu et al 2011, James et al 2010
TNFRSF11A	Codes for a member of the TNF-receptor superfamily. Like *PLCG1* , is also involved in mast cell activtion. It also plays an important role in defense against pathogens, and blood–brain barrier function	rs1805034	C>T	Allele T is associated with an increased risk of urticaria and Angioedema in patients who are treated with acetaminophen: patients with CC/CT genotypes are at higher risk compared to patients with the TT genotype.	Ayuso et al 2015, Polyzoidis et al 2015
UGT1A gene cluster	Responsible for glucuronidation in phase II metabolism which accounts for approximately 40% to 70% of xenobiotic elimination	rs10929303	T>C	Allele C confers faster glucuronidation, which increases water-solubility of the chemicals	Mehboob et al 2017
		rs8330	G>C		
		rs1042640	G>C	Patients with the CC genotype may have an increased risk of liver failure due to unintentional acetaminophen overdose as compared to patients with the CG or GG genotype.	Court et al 2013
UGT2B15	Main *UGT* responsible for bisphenol A glucuronidation	rs1902023	A>C	Allele C confers reduced metabolism to *UGT2B15*	Hanioka et al 2008 & 2011
VDR	Vitamin D receptor	rs731236	T>C	This polymorphism might alter *VDR* mRNA levels through regulation of mRNA stability and therefore affect its function	Cieslinska et al 2017, Fei et al 2016

4.2 ELEAT RESULTS

The population sample analyzed included 20 children with an age range from 6 to 12 years old (mean age of 9,1), and a higher percentage of males (65%), in line with the male to female ratio in ASD. All children met criteria for ASD according to the DSM V. Furthermore, as seen in figure 3, some children (4) also presented a number of comorbidities such as speech or language disorder, developmental delay, intellectual disability, social communication disorder, epilepsy and attention deficit hyperactivity disorder.

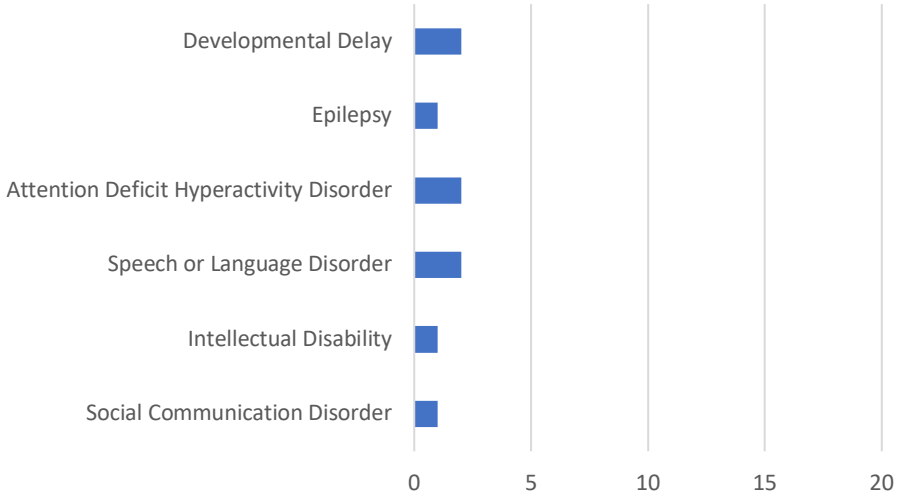


Figure 3 Comorbidities present in group of probands

The probands' biological mothers filled out the ELEAT. Parents' age at probands birth ranged from 22 to 38 years in mothers (mean age 29,7 years), and 24 to 40 years in fathers (mean age 32,7 years). As illustrated in figure 4, mothers' education level ranged from *2º cycle* to Post-Graduate, with the highest percentage (40%) having completed a bachelor's degree. Father's education level ranged from *1º cycle* to Bachelor's degree, with the highest percentage (60%) having completed High-School.

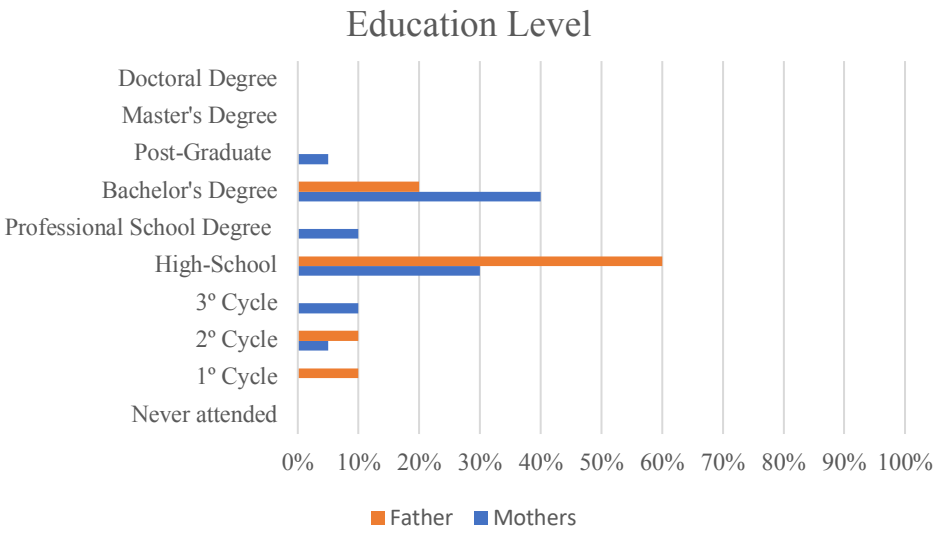


Figure 4 Parent's education level

Total household income (as seen in figure 5) fluctuated between less than 800 € per month to more than 2699 € per month, with the highest percentage (30%) falling on 800€ to 1499€ per month category. Some mothers chose not to answer this question.

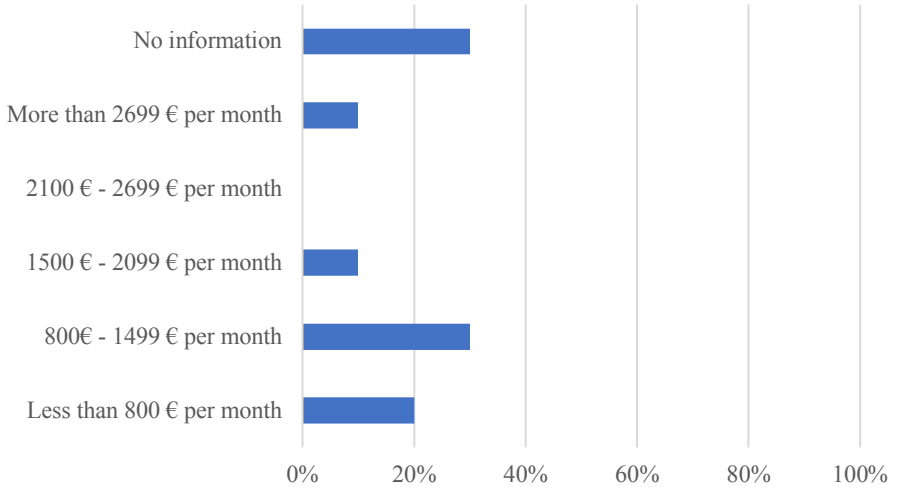


Figure 5 Total household income

Below the most relevant results for each module are highlighted.

Results for module B (Breastfeeding and Child Diet), highlight that most infants where both breastfed (80%) and also given substitution milk (74%), with the majority of the mothers opting for cow derived substitution milk (71%). 70% of probands were fed with clear rigid plastic bottles. Of these, 4 out of 14 contained BPA, while 9 out of 14 mothers did not know whether the plastic bottles contained BPA.

Module M (Maternal Condition/ Medical Interventions) results show that only a moderate number of mothers had any kind of medical conditions or medical procedures and took medication during the period covered by the ELEAT. The highest percentages were: 36% of mothers were given oxytocin to speed labor process, 30% had dental cleanings or other dental procedures, 20% suffered from an influenza infection during pregnancy, 25% used prescription medication for pain, fever or inflammation during pregnancy and 20% took female hormone medication. There was not a high use of pharmaceutical drugs reported by the mothers. Howerever, Ben-u-ron (20%) and Paracetamol (15%) were the most frequently used, while Nausefe, Atosiban, Primperan, Lamotrigine, Clavamox, Buscopan, Atarax, Valium, Antibiotics, Kompensan S, Gino-Canesten, Flagyl Ovule, Cerazette and Harmonet were each taken only once (5%).

Regarding food consumption in Module D (Maternal Diet) we analyzed how often mothers ate cheese, eggs, fish and red meat. Results show that the most frequent consumption rate was that of 1-2 times per week for cheese (25%), eggs (30%), red meat (30%), and fish (35%), with the most consumed fish being horse mackerel (40%), gilthead bream (35%), hake (35%) and dried cod (30%). However, some mothers reported to never eating cheese (10%), eggs (10%) and fish (5%). Additionally, as seen in figure 6, a meaningful percentage of mothers (65%) consumed bottled water during and after pregnancy.

In terms of supplements (also illustrated in figure 6), results of module S (Supplements) highlight that mothers mainly took folic acid (80%), iron (70%), vitamins or supplements containing folic acid (65%), prenatal vitamins (45%), where 30% also contained iron, and finally, calcium supplements (32%). On the

other hand, there was no reported consumption of vitamin A or retinal, beta carotene, vitamin E, niacin, selenium, zinc, fish oil or omegae3 fatty acids, gingko biloba, St. John's Wort, co-enzyme Q-10, or probiotics.

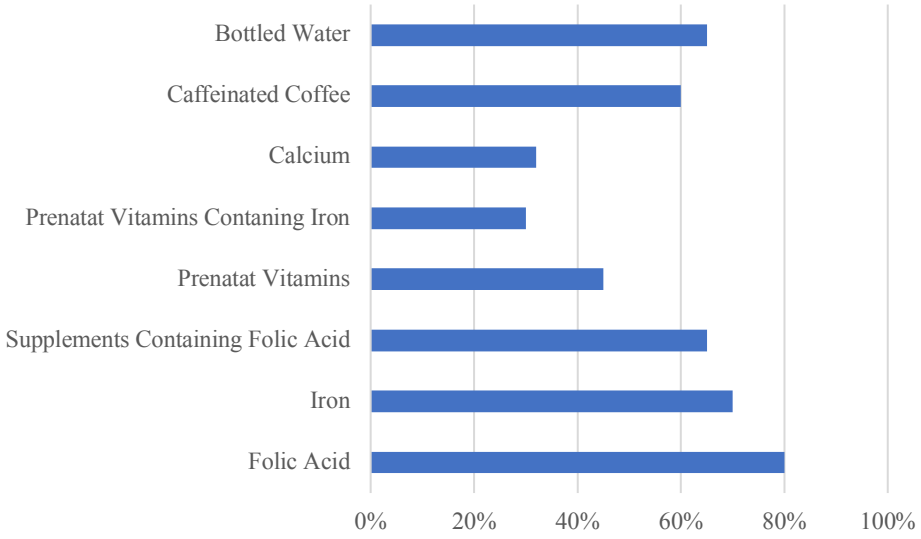


Figure 6 Some of the products consumed during the period covered by the ELEAT

In Module L (Lifestyle), 45% of mothers answered the question regarding sunlight exposure. From the hours reported by the mothers, it was calculated that an average of 6.2 hours per week were spent outside, and an average of 3.6 of these hours were during sunny periods of the day. Regarding caffeine, also shown in figure 6, a significant percentage of mothers (60%) reported consuming caffeinated coffee. Of these, 100% reported consuming it before pregnancy, more than a half (66%) during pregnancy and (50%) while breastfeeding. Regarding smoking and consumption of recreational drugs, none of the mothers consumed electronic cigarettes or any tobacco or nicotine products, alcohol, marijuana or any other recreational drugs. On the other hand, regarding second hand smoke, 30% reported that the biological father smoked cigarettes during the three months before conception, and 47% reported that they lived with someone who smoked cigarettes. However, of these, the majority (66%) reported that the cigarettes were always smoked outside their homes. In addition to this, no one living in the mothers' homes smoked any other products anywhere inside the house. Hence the exposure to secondhand smoke was low, and there was little evidence for direct exposure to cigarette smoke.

Results of Module H (Home Environment) highlight that almost half of the mothers (45%) drank tap water, and the majority did not filter it in any way (66%). With respect to home-derived pollutants, fireplace was described to be the main heating source at home (35%), and the majority of the mothers used a gas stove (85%). However, 79% reported having a fume fan over the stove, and 87% of these indicated that they used it all/most of the time. Approximately one third of mothers (32%) answered affirmatively to having any mold or mildew on walls or other surfaces other than in the shower or bathtub. Concerning the house location relative to pollution sites, more than half of the mothers (53%) lived within ~400 meters of a busy road/highway, some lived within ~400 meters of an agricultural field or golf course (37%), but none lived within ~400 meters of a landfill.

In module E (Environment), few mothers (40%) answered question relative to the potential exposure to PBDEs, namely regarding the number of stuffed furniture owned as this chemical is used in the polyurethane foam. It was calculated that, while pregnant, mothers owned an average of 5.6 pieces of furniture with cushions at home such as sofas and chairs with cushions. In addition to this, three months prior to becoming pregnant through the end of the first year after, over half of the mothers installed new carpets or rugs (60%). Lastly, 35% of the mothers reported painting/varnishing walls, ceilings, or furniture inside their homes.

Concerning pesticide use, only a very low percentage of mothers (ranging from 5% to 16%) used any type of pesticides inside or outside their homes.

In relation to the amount of times certain cosmetic products were used by the mothers, the most frequent rate of usage was 1 to 2 times per day, during pregnancy and during the child's first year, for products such as: deodorant (65% and 63% respectively), lotion (60% and 58% respectively), liquid soap (50% and 42% respectively), and perfume (37% and 33% respectively). Nonetheless, hair gel, hair spray, and nail varnish/polish were not frequently used.

When examining occupational exposures in Module O (Occupation and Exposures), as seen in figure 7, results indicate that there was a very low rate of maternal exposure during pregnancy. The highest, but moderate, percentage of exposure was that of petrol exhaust, where 20% of mothers were exposed during pregnancy. No pattern was found in maternal or paternal occupation.

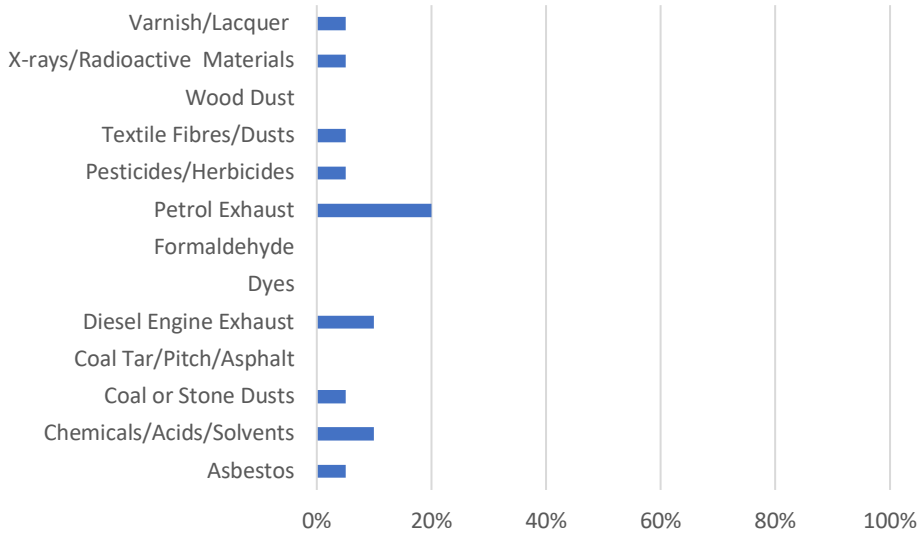


Figure 7 Mother's Occupational Exposure During Pregnancy

The last module of the tool, Module I (Instrument Rating), consists of questions were the mothers rate the instrument. Concerning the length of the questionnaire, 45% of the mothers considered it a little too long, while 45% considered it much too long. In relation to questions regarding medical history, breastfeeding, diet, vitamins/supplements and lifestyle/substance use, between 85% and 53% of the mothers reported high certainty concerning their given answers. Only in questions regarding occupations/exposures and environment did the mothers express slightly lower confidence about their answers, with 42% and 47%

reporting high certainty, respectively. Notably, the latter questions are part of the last two module to be answered. When evaluating the questionnaire as a whole, 68% of the mothers found that the instructions to answer the tool were clear, and 53% felt sure about their answers. Additionally, 61% believed that the questions asked were important. Regarding the online questionnaire, the feedback was very positive. Mothers agreed that it was easy to understand the instructions (80%) and to navigate through questions on the survey (93%).

For more detailed results, please refer to attachment S4, following each module.

4.3 Genotyping Results & Exposure

Out of the 20 participants who filled the ELEAT, we were able to obtain 16 biological samples: 9 blood samples and 7 buccal swab samples. However, after quantifying each buccal swab sample using the NanoDrop Spectrophotometer, we chose to exclude 2 participants from this genotyping, as the DNA obtained was degraded (absorbance at 260nm and 280 nm – A260/280 ratio – was below the normal values 1.80-2.00). Therefore, 14 DNA samples were used for the genotyping.

Individuals were genotyped for particular genes according to environmental liability during early development. In this way, patients exposed to acetaminophen were screened for polymorphisms in genes *UGT1A*, *PLCG1*, and *TNFRSF11A,* and patients exposed to oxytocin were screened for the polymorphism in gene *OXTR* (table 7).

Table 7. Genotyping results for probands whose mothers took acetaminophen, and for those whose mothers took oxytocin. Listed are the polymorphisms relevant for each drug. –: sequencing results had low quality and therefore could not be read.

Subject Number	Acetaminophen			
	UGT1A	***PLCG1***	***TNFRSF11A***	Exposure
	rs1042640	rs2228246	rs1805034	
Subject 8	GC	AA	--	*yes*
Subject 12	GC	AA	CT	*yes*
Subject 13	GG	AA	CT	*yes*
Subject Number	**Oxytocin**			
	OXTR		Exposure	
	rs2268498			
Subject 2	CC		*yes*	
Subject 4	CC		*yes*	
Subject 10	--		*yes*	
Subject 13	TC		*yes*	
Subject 14	TT		*yes*	

All 14 individuals were genotyped for *MTHFR, CYP2R1, VDR, AHR, UGT2B15, GSTM1, PON1, ACHE, ABCB1, CYP3A4, CYP2C19,* and *CYP2D6* (table 8) which are involved in the activity of various environmental factors such as folic acid, vitamin D, PAHs, BPA, organophosphate pesticides, heavy metals, and pharmaceutical drugs. Due to technical difficulties, we were unable to genotype polymorphisms in *CYP1A1*, *SLC19A1*, and *UGT1A* gene cluster. Electrophoresis gel results for the amplification of fragments containing the screened polymorphisms are shown in attachments S5 and S6, while the ones containing digestion patterns and *GSTM1* genotyping are shown in attachments S7 and S8 respectively. An example of Sanger Sequencing results, for rs1854560 polymorphism of *PON1* gene, is shown in attachment S9.

We observed that some probands carried the minor allele of polymorphisms in the following genes*: CYP2R1, VDR, AHR, GSTM1, UGT2B15, PON1, ACHE, ABCB1, CYP2C19* and *CYP3A4*. We also noticed that certain probands carried more than one genetic variant, in genes involved in the activity of different environmental factors. Taking into account that the frequency of the minor allele for rs1801133 of *MTHFR* is approximately 0.3397 in the European Non-Finnish population, it was surprising to note that it was not represented in our sample. Therefore, to ensure that our results were valid, we tested the HinfI enzyme used in digestion by selecting a DNA fragment from a known heterozygous subject for the polymorphism. Results showed that the enzyme was functional.

Furthermore, we individually evaluated probands' exposure by analyzing mothers' answers to questions selected for each polymorphism (as seen in table 5). We established probands' exposure to the environmental factor being analyzed according to: whether the mothers answered positively to questions regarding the specific environmental factor, or if they selected an answer option that potentially conveyed exposure to the specific environmental factor. On the other hand, in some cases we did not have enough information (e.g.: some mothers did not answer) to conclude that probands were exposed to the xenobiotic, but we also could not conclude that they were not exposed to it. Therefore, we chose the label "don't know" instead of "no". This was predominantly the case for variants involved in heavy metal and pesticide activity, as most of the questions regarding these exposures did not provide sufficient information to conclude exposure. For example, question 33 of Module E regarding exposure to heavy metals asked "was the blood of the child of interest tested for lead when he/she was young?". Where half of mother answered "no" while the other half answered "don't know". Additionally, as seen in answers to question 11 in Module H, no mother reported having their drinking water tested for chemicals such as pesticides, lead, arsenic, nitrates, etc. Furthermore, although mothers did responded that they did not use pesticides directly, the information gathered from this group of questions are not sufficient to conclude that there was no exposure to these chemicals.

Table 8. Genotype results for all probands who were screened for polymorphisms in genes involved in the processing of different environmental factors, and their exposure to each one of these environmental factors. Minor alleles are represented in red. - -: sequencing results had low quality and therefore could not be read.

Subject Number	Folic Acid		Vitamin D			PAHs		Multiple xenobiotics		Bisphenol A	
	MTHFR	Exposure	***CYP2R1***	***VDR***	Exposure	***AHR***	Exposure	***GSTM1***	Exposure	***UGT2B15***	Exposure
	rs1801133		rs10741657	rs731236		rs4410790		GSTM1*0		rs1902023	
Subject 1	CC	*Yes*	AA	TC	*Yes*	--	*Yes*	GSTM1 +/-	*Yes*	AA	*Don't Know*
Subject 2	CC	*Yes*	AA	TC	*Yes*	TC	*Don't Know*	GSTM1 +/+	*Yes*	AC	*Don't Know*
Subject 3	CC	*Yes*	AA	TC	*Yes*	TC	*Yes*	--	*Yes*	--	*Yes*
Subject 4	CC	*Yes*	AA	TT	*Yes*	TC	*Don't Know*	GSTM1 +/+	*Yes*	CC	*Don't Know*
Subject 5	CC	*Yes*	AA	TT	*Yes*	TC	*Don't Know*	GSTM1 +/-	*Yes*	AC	*Yes*
Subject 6	CC	*Don't Know*	AA	TT	*Yes*	--	*Yes*	GSTM1 -/-	*Yes*	CC	*Don't Know*
Subject 7	CC	*Yes*	AG	TT	*Yes*	TC	*Don't Know*	GSTM1 +/+	*Yes*	AC	*Yes*
Subject 8	CC	*Yes*	AA	TT	*Yes*	--	*Don't Know*	GSTM1 +/-	*Yes*	AC	*Yes*
Subject 9	CC	*Yes*	AG	TC	*Yes*	CC	*Yes*	GSTM1 +/+	*Yes*	CC	*Yes*
Subject 10	CC	*Yes*	AG	TT	*Yes*	CC	*Yes*	GSTM1 +/+	*Yes*	AC	*Yes*
Subject 11	CC	*Yes*	AG	TC	*Yes*	TT	*Yes*	GSTM1 +/+	*Yes*	AC	*Yes*
Subject 12	CC	*Yes*	AG	TT	*Yes*	TC	*Yes*	--	*Yes*	AC	*Yes*
Subject 13	CC	*Yes*	AA	TT	*Yes*	TC	*Yes*	GSTM1 +/-	*Yes*	AC	*Yes*
Subject 14	CC	*Yes*	AA	TT	*Yes*	CC	*Yes*	GSTM1 -/-	*Yes*	AC	*Yes*

Subject Number	Organophosphate pesticides				Heavy metals		Multiple pharmaceutical drugs & xenobiotics				
	PON1		***ACHE***	Exposure	***ABCB1***	Exposure	***CYP3A4***	***CYP2C19***	***CYP2D6***		Exposure
	rs854560	rs662	rs2571598		rs1045642		rs2740574	CYP2C19*2	CYP2D6*4	CYP2D6*6	
Subject 1	AA	CC	CT	*Don't Know*	GG	*Yes*	TT	GG	--	--	*Don't Know*
Subject 2	--	TT	--	*Yes*	GG	*Don't Know*	--	GG	--	--	*Yes*
Subject 3	AT	--	CC	*Don't Know*	GG	*Yes*	TT	GG	--	--	*Yes*
Subject 4	TT	TT	CC	*Yes*	GG	*Don't Know*	TT	GG	--	--	*Yes*
Subject 5	AA	TC	CC	*Don't Know*	GG	*Don't Know*	--	--	--	--	*Yes*
Subject 6	AT	--	--	*Don't Know*	GG	*Don't Know*	--	--	CC	AA	*Don't Know*
Subject 7	AT	TT	CC	*Don't Know*	GG	*Don't Know*	TT	GG	CC	AA	*Don't Know*
Subject 8	AA	TC	TT	*Yes*	GG	*Don't Know*	TT	GG	CC	AA	*Yes*
Subject 9	AT	TC	CC	*Yes*	GG	*Don't Know*	TT	GG	CC	AA	*Yes*
Subject 10	AT	TT	CC	*Don't Know*	GA	*Don't Know*	TT	GA	CC	AA	*Yes*
Subject 11	AA	TC	CT	*Don't Know*	GG	*Don't Know*	TC	GG	--	--	*Yes*
Subject 12	AA	TC	CT	*Don't Know*	GG	*Don't Know*	TT	GG	--	--	*Yes*
Subject 13	TT	TC	CT	*Don't Know*	GG	*Don't Know*	TT	GA	CC	AA	*Yes*
Subject 14	AA	CC	CT	*Yes*	GG	*Don't Know*	TT	GG	CC	AA	*Yes*

5. DISCUSSION

There has been an accumulation of evidence pointing to the role played by environmental factors in ASD risk, such as the effects on neurodevelopment of endocrine disruptive chemicals like xenobiotics that affect metabolism and cross physiological barriers. As mentioned in the introductory part of this work, environmental risk may only account for a percentage of the total risk of ASD. A substantial contribution is given by genetic variants which interact at various levels with different environmental factors, giving rise to brain disturbing pathways and ASD phenotypic manifestation. This work set to explore and study gene-environment interactions between several candidate genes and environmental factors. On doing so, we applied an environmental exposure questionnaire (ELEAT) and explored the presence of polymorphisms in a total of 18 candidate genes, in a sample of 14 individuals diagnosed with ASD. We aimed to detect variants relevant to organism's response to disruptive exogenous factors. From the questionnaire's answers, we observed that probands were indeed exposed to a number of environmental factors previously associated to ASD. Additionally, genotyping results indicate that offspring of this group of mothers carry variants in genes involved in the functioning of such factors. Most of these variants have not been previously associated with ASD, but have the potential to contribute to the disorder's onset.

The gender ratio found in this study is in accordance with male prevalence bias, seeing as 65% of the probands were male, and is thus representative of the general ASD population. ELEAT results suggest that the majority of the infants were both breastfed as well as given cow derived substitution milk. Consumption of dairy products has been of particular concern due to the amount of chemical contaminants that may be introduced during milk production, dairy processing or packaging (Jahed Khanik, 2007). It has been suggested that most of these contaminants are veterinary drugs such antibiotics, steroid hormones, bovine growth hormone, parasiticide drugs, as well as pesticides and heavy metals (Jahed Khanik, 2007). Exposure to such chemicals may have detrimental effects on infants seeing as they do not have a fully developed immune system. Indeed breastfeeding mothers can pass such contaminants directly to infants through breast milk. Studies have found concerning levels of EDCs in mothers' colostrum (Kalkbrenner et al., 2014; Yi et al., 2013), it is therefore safe to assume that infants that are breastfed and bottle fed at the same time may have an increased risk of being exposed.

Furthermore, results suggest that a significant number of probands were fed with clear rigid plastic bottles, which have been recognized as a source of BPA. Four mothers confirmed that the feeding bottles did contained BPA while others didn't know if this chemical was present in the bottles. It is important to note that the fact that some mothers did not know may be attributable to forgetfulness, or mainly because the general population is not familiar with such terminology. Therefore, the number of children exposed to this EDC due to the use of plastic bottles may be greater than reported. In addition to this, a significant number of mothers reported consuming bottled water during and after pregnancy, which is also a BPA source. One of the polymorphisms screened in probands was rs1902023, whose allele C confers reduced metabolism activity to *UGT2B15*, a gene of high importance seeing as it is the predominant isoform of BPA glucuronidation in humans in phase II metabolism (Hanioka, Oka, Nagaoka, Ikushiro, & Narimatsu, 2011; Hanioka, Naito, & Narimatsu, 2008). Genotyping results revealed that 9 probands were heterozygous (AC), while 3 were homozygous for the minor allele (CC). Additionally, when analyzing exposure, results indicate that one of the homozygous probands was certainly exposed to BPA. Considering that this individual could not correctly metabolize it, the exposure to this chemical may have had negative

consequences during development as unmetabolized BPA would have accumulated and reached toxic levels.

In regards to pesticides, there was a very low use of these chemicals by the mothers, indoors or outdoors. Pesticides are widely applied in agriculture and on livestock, and residues are able to infiltrate through the soil into surface water because of their water solubility (Lionetto, Caricato, Calisi, Giordano, & Schettino, 2013; Diamanti-Kandarakis et al., 2009). Therefore, we cannot exclude exposure completely during pregnancy and through the child's first year of life. Recent studies detected pesticides in food, ground and drinking water, natural surface waters, and marine organisms (Lionetto et al., 2013). It is important to note that some mothers reported living near a golf or agriculture field. We genotyped probands for polymorphisms rs854560 and rs662 in the *PON1* gene, which encodes the enzyme paraoxonase, involved in the inactivation of organophosphate pesticides in humans. The alternative alleles T and C respectively, have been linked to decreased paraoxonase activity against OP compounds, and associate with autism vulnerability (D'Amelio et al., 2005). We observed that, for rs854560, 5 probands were heterozygous (AT), while 2 were homozygous (TT) for the minor allele. Additionally, 6 probands were heterozygous (TC), while 2 were homozygous (CC) for the minor allele in rs662. Probands also carried polymorphisms in *AChE*, a key enzyme in the nervous system which hydrolyzes the neurotransmitter acetylcholine. This enzyme is essential for the normal functioning of the nervous system as it terminates synaptic transmission, preventing continuous nerve firings at nerve endings. In addition to this, it is the primary target of inhibition for organophosphorus pesticides, which bind to the enzyme and inactivate it (Lionetto et al., 2013). When screened for polymorphism rs2571598, 5 probands were heterozygous (CT) while 1 was homozygous (TT) for minor allele. Since this enzyme is targeted by pesticides, individuals who carry this polymorphism are even more sensitive to these chemicals, seeing as the alternative allele (T) confers reduced enzymatic activity and has been associated with reduced serum *AChE* levels (Reale et al., 2018). Interestingly, previous studies have already shown that individuals with ASD carry potentially pathogenic SNVs within the ACHE gene (Iossifov et al., 2015). It is important to highlight that individuals such as subject 13, which carrry the minor allele of all three polymorphisms (heterozygous for both rs662 and rs2571598 on *PON1*, and homozygous for minor allele in rs854560 of *AChE*), would probably have been highly susceptible to pesticides, and therefore exposure should have been avoided as much as possible. The exposure status of this individual is unknown since the mother answered negatively to questions regarding the use of pesticides. However, subject 13 may have been exposed by maternal consumption of contaminated foods or water during pregnancy and/or while breastfeeding. Thus, the ELEAT does not provide enough information, and other sources of information like dried blood spots collected at birth or deciduous teeth might be very useful to directly measure exposure.

Regarding tobacco, although both direct and secondhand smoke are complex mixtures including nicotine, volatile organic compounds, and metals such as cadmium and lead, evidence does not support a strong causal link between direct maternal smoking during pregnancy and autism (Kalkbrenner et al., 2014).Tobacco smoke also contains PAHs (Moorthy, Chu, & Carlin, 2015), one of the EDC compounds. Results, showed no direct exposure of our probands as none of the mothers smoked during pregnancy. However, there is evidence that exposure to secondhand smoke may disrupt neurodevelopment (Kalkbrenner et al., 2014). Some mothers reported that the biological father smoked cigarettes during the three months before conception, and others reported that they lived with someone who smoked cigarettes. It is important to note that, more than half reported that smoking took place outside the house, thus reducing contact with secondhand smoke. Residential proximity to freeway has also been associated with exposure

to traffic-related air pollutants such as heavy metals, diesel compounds (Tordjman et al., 2014; Lyall et al., 2014) and PAHs (Moorthy et al., 2015), it is important to note that over half of the mothers reported living within ~400 meters from a busy road/highway. Genotyping results for rs4410790 in *AHR* revealed that 7 probands were heterozygous, while 3 were homozygous for the minor allele (CC). *AHR* is a receptor that is activated by ligands such as PAHs, and in turn activates cytochrome P450 enzymes which metabolize this xenobiotic. PAHs are metabolized by these and other enzymes into reactive metabolites, which can elevate reactive oxygen species (ROS) and react with DNA (Moorthy et al., 2015). Rs4410790 has thus far only been associated with higher coffee consumption and lower plasma caffeine levels (Cornelis et al., 2014). However, this may be the result of higher ARH activity, leading to a higher expression of cytochrome P450 enzymes responsible for the metabolization of caffeine. Following this rationale, higher *ARH* activity would also lead to higher expression of cytochrome P450 enzymes involved in the activation of PAHs. Therefore, individuals such as subject 9, 10 and 14 who are homozygous for the minor allele (CC), may be prone to the buildup of reactive PAH metabolites if phase II enzymes do not have the same capacity in clearance. Interestingly, subject 14, who was exposed to PAHs during development, is also homozygous for GSTM1*0 (deletion of GSTM1 in both alleles, will be further discussed), leading to an impaired phase II metabolism. This exposure may have led to detrimental effects in neurodevelopment.

The *ABCB1* gene encodes for P-glycoprotein, responsible for the protective functional properties of the blood-brain barrier (Møllgård, Dziegielewska, Holst, Habgood, & Saunders, 2017; Brambila-Tapia, 2013). This protein is also abundant in the placenta, and was the first discovered to offer fetal protection against toxicity (Walker et al., 2017). When exposed to heavy metals, and reactive oxygen species, the expression of P-glycoprotein is upregulated (Miller, Bauer, & Hart, 2008). We examined whether probands were carriers of the causative alle in rs1045642, which has been associated with decreased P-glycoprotein expression in tissues studies, including placenta (Brambila-Tapia, 2013). Our results revealed that 1 proband was heterozygous for this polymorphism (GA). Such genotype may have conferred a higher sensitivity to heavy metals, both during and after embryonic development. This individuals' exposure is not clear seeing as we don't have enough information from the mothers.

The use of cosmetic products is widespread in the general population. However, studying the use of such products (e.g.: lotions and perfumes) in pregnant women is important as they can be a source of phthalate, which have also been recognized as EDCs (Lyall et al., 2014; Diamanti-Kandarakis et al., 2009; Kalkbrenner et al., 2014). Interviewed mothers did not completely exclude these products from daily routine during pregnancy, with the most frequently used cosmetic products being deodorant, lotion and liquid hand soap. We can therefore conclude that the children may have been exposed to some levels of phthalates.

In terms of home environment, it was estimated that, while pregnant, mothers owned an average of 5.6 pieces of furniture with cushions at home such as sofas and chairs with cushions, and over half reported installing new carpets or rugs three months prior to becoming pregnant through the end of the infant's first year of life. Stuffed furniture is a potential source of PBDEs a compound used in polyurethane foam in furniture. These chemicals are released into the environment over time since they are not chemically bound to the materials in which they are used (Castorina et al., 2011; Kalkbrenner et al., 2014). The same principle applies to carpets, which are also a source of PBDE (Kalkbrenner et al., 2014). A study found that women having 3 or more pieces of stuffed furniture in their home had higher serum PBDE levels (Castorina et al., 2011). Following this criteria, it can be presumed that interviewed mothers were living in an space contaminated with PBDEs, and therefore were exposed to these chemicals during pregnancy. This is worrying as this class of chemicals have been recognized as EDCs, which disrupt hormone signaling and

have been linked with increased risk of ASD onset. It has been speculated that PBDEs do this by disrupting the normal function of thyroid hormone, because they are structurally similar (Diamanti-Kandarakis et al., 2009; Kalkbrenner et al., 2014). It has also been suggested that individuals with ASD show an abnormal immune activation when exposed to PBDEs when compared to controls (Koufaris & Sismani, 2015). An additional concerning factor, is that breast milk has been identified as a source of PBDEs exposure (Castorina et al., 2011; Kalkbrenner et al., 2014), as well as in cord and placental tissue (Castorina et al., 2011).

We genotyped probands for polymorphisms in *GSTM1* and *UGT1A*, both involved in phase II metabolism of multiple xenobiotics including phthalates and PBDEs. In normal physiological conditions, glutathione reductase enzyme activities are able to sustain a high total plasma glutathione level versus oxidized glutathione redox ratio (James et al., 2006). However, excessive oxidative stress can disturb this balance and result in oxidized glutathione being transported into the plasma, which has been observed in autistic children (James et al., 2006; Buyske et al., 2006). The variant examined in this pilot study is the deletion of *GSTM1* (GSTM1*0), which results in reduced activity depending on the number of alleles deleted. Our results showed that 4 probands were heterozygous, while 2 were homozygous for GSTM1*0 (*GSTM1* was deleted in both alleles). This deletion has been thought to contribute to the onset of some cases of autism, due to the failure in detoxifying important compounds, including some that could be agents or products of oxidative stress, thus affecting brain development during gestation (Buyske et al., 2006). Therefore, probands who carry both deletions will be highly susceptible to a number of xenobiotics metabolized by glutathione S-transferase enzyme, such as phthalates and PBDEs. Results from the ELEAT show that both individuals were exposed to some of these chemicals.

Genes from the *UGT1A* cluster also play a role in phase II metabolism: they glucuronidate and eliminate several exogenous compounds by turning lipophilic molecules into more water soluble molecules than can be easily excreted. Individuals homozygous for alleles C in both rs10929303 and rs8330 are characterized as fast glucuronidators, while individuals homozygous for alleles T and G, respectively, are considered slow glucuronidators (Mehboob et al., 2017). However, as genotyping was not successful, we were unable to screen for these polymorphisms in our group of probands.

Overall, the incapacity to properly metabolize endocrine disruptive chemicals can lead to harmful consequences in neurodevelopment, seeing as these xenobiotics are known to disrupt hormone function, which are involved in developmental processes. It is important to note that the general population is exposed to some level of these chemicals daily. However, these are specially harmful for pregnant women as most of these chemicals are able to cross the physiological barriers. This is even more detrimental when fetuses carry polymorphisms such as the ones mentioned above which make them more susceptible and can lead to serious reactions when exposed to such EDCs, even in lower doses. This process could result in increased risk of ASD (Koufaris & Sismani, 2015)

ELEAT results demonstrate that there was a regular intake of iron and folic acid. Multiple studies have associated prenatal intake of folic acid with a decreased risk of ASD development (Lyall et al., 2014; Tordjman et al., 2014; Wang et al., 2017). Taking into account that studies have indicated that variants on genes involved in processing folate, such as *MTHFR* and *SLC19A1*, which are both involved in placental function (Carter, 2016), are common in children with ASD (James et al., 2010; Schmidt et al., 2012), we screened probands for SNPs in both genes. Results for polymorphism rs1801133 of *MTHFR* gene showed that probands were all homozygous for the wildtype allele (CC). No proband carried allele T, which has been related to reduced enzyme *MTHFR* activity (James et al., 2010; Liu et al., 2011; Schmidt et al., 2012).

This allele has been associated with increased folate requirement and elevated levels of homocysteine, with the latter being linked to negative health effects (James et al., 2010; Schmidt et al., 2012). Therefore, the study of this polymorphism is important seeing as mothers pregnant with children with SNPs such as this require higher amounts of folate for proper neurodevelopment (Schmidt et al., 2012). Due to technical difficulties, we were not able to successfully genotype probands for polymorphism rs1051266 of *SLC19A1*. Allele G of this SNP has been linked to reduced folate carrier protein, and therefore low plasma folate levels (James et al., 2010). Studying the accumulative effect of both these polymorphisms is very important considering that a 3-fold increase autism susceptibility has been associated with the combination of homozygous genotype (GG) for the alternative allele in rs1051266, and genotypes (CT/TT) in rs1801133 (James et al., 2006).

Low levels of vitamin D have been associated with risk of ASD and other developmental problems (Vinkhuyzen et al., 2017; Rebecca J. Schmidt et al., 2015; Cieślińska et al., 2017; Chen et al., 2016). Vitamin D can be obtained by exposure to sunlight, and also by the consumption of foods such as eggs, meat and fatty-fish (De-Regil, Palacios, Lombardo, & Pena-Rosas, 2016). ELEAT results indicate that mothers spent an average of 3.6 hours per week outside during sunny periods of the day. Although the World Health Organization supports sunlight exposure, they recognize that the recommended amount of time spent in the sun by pregnant women may vary due to certain factors: amount of skin exposed, time of day, latitude and season, sunscreen use, skin pigmentation, etc. (http://www.who.int/elena/titles/guidance_summaries/vitamind_supp_pregnancy/en/). Therefore, without a control group, we cannot reach any conclusions from this value. Additionally, most mothers consumed foods known to be a source of vitamin D. One of the most consumed types of fish was horse mackerel (40%), a type of fatty-fish that is rich in this nutrient. When investigating the presence of polymorphisms in *CYP2R1* and *VDR*, which are both involved in processing vitamin D, we found the presence of the minor allele of both genes. Allele G in polymorphism rs10741657 of *CYP2R1* has been associated with lower circulating levels of 25(OH)D and an increased risk of ASD (Rebecca J. Schmidt et al., 2015), while allele A in polymorphism rs731236 in *VDR* may be correlated with development of ASD symptoms (Cieślińska et al., 2017). It has been implied that individuals with *VDR* variants require higher levels of vitamin D considering that they might have different activation threshold compared to carriers of the wildtype allele (Cieślińska et al., 2017). We observed that 5 probands were heterozygous for rs10741657, while 5 were heterozygous for rs731236. Both polymorphisms affect function of these genes, and thus result in the incorrect activity of this vital vitamin, individuals such as subject 9 and 11 which are heterozygous for both polymorphisms, may be highly pre-disposed to vitamin D deficiency. Therefore, it can be presumed that, although mothers did expose fetuses to vitamin D, mothers of probands 9 and 11 would need higher doses of this vitamin in order to promote a healthy neurodevelopment.

Only a moderate number of mothers reported having any kind of medical conditions or using pharmaceutical drugs during the period covered by the ELEAT. A thorough investigation of this area is vital for ASD research, as developing fetuses may be exposed to pharmaceutical drugs that they cannot completely metabolize due to an undeveloped detoxification system (Shao, Stapleton, Lin, & Gallagher, 2007) and are believed to have a heavier body burden compared to adults (Yi et al., 2013). A study has shown that, while fetal liver hematopoietic stem cells have an active phase II metabolism, due to high Glutathione *S*-Transferase expression, they lack a fully functional phase I metabolism. This is because *CYP1A1, CYP2E1, CYP3A4,* and *CYP3A5* were expressed at low levels, while *CYP1A2* and *CYP3A7* were not detected (Shao et al., 2007). These results suggest that fetus metabolism is only partially functional.

This is important for drug metabolization as cytochrome p450 enzymes play a crucial role in the drug metabolism: families 1-3 are responsible to 70 to 80% of all phase I dependent drug metabolism (Wijnen et al., 2007). Therefore, low levels of cytochrome p450 expression is fetuses make them more susceptible to medication taken by mothers during pregnancy.

From the medications taken by mothers who filled the ELEAT, Paracetamol, Ben-u-ron, Atosiban, Primperan, Lamotrigine, Clavamox, Atarax, Valium, Flagyl ovule and antibiotics all have active ingredients that are able to cross the placenta (Bremer et al., 2017; Valenzuela, Craig, Bernhardt, & Holland, 1995; Sørensen et al., 2000; Myllynen, Pienimäki, & Vähäkangas, 2003; Iqbal, Sobhan, & Ryals, 2002; Koss et al., 2012; Pacifici, 2006). Furthermore, Amoxicillin, Acetaminophen and Diazepam, which are some of the active ingredients taken by the mothers and able to cross the placenta, are also listed as being part of the top 200 most frequently consumed medications in Portugal. This fact is imperative as it indicates a high rate of consumption of chemicals with the potential to disrupt fetus development.

Following the hypothesis that maternal drug metabolization is altered during pregnancy, thus increasing the expression of certain metabolism enzymes such as *CYP2A6*, *CYP2B6*, *CYP3A4* and *UGT1A4* (Jeong, 2010), a higher dosage of pharmaceutical drugs metabolized by these enzymes may be necessary to reach the level required for treatment. However, it is plausible that the fetus will somewhat be exposed to a higher dosage of the medication before the over expressed maternal cytochrome p450 enzymes can lower its concentration. This implication should be considered as it may be of particular importance to fetus that carry genetic variants for low metabolizers in these enzymes, and therefore have a reduced capacity to metabolize such pharmaceutical drugs.

A large portion of mothers reported consuming caffeinated coffee. A potential role played by caffeine in ASD onset has not been explored. *CYP1A2* encodes for the main enzyme responsible for phase I metabolization of caffeine (Sulem et al., 2011). The fact that *CYP1A2* expression decreases in mothers during pregnancy (Jeong, 2010), and is not detected in hematopoietic stem cells during fetal development (Shao et al., 2007) should be considered. Future studies should analyze polymorphisms in this gene in the mother.

In our study, probands were genotyped for SNPs in *CYP3A4*, *CYP2D6* and *CYP2C19*. *CYP3A4* is a fundamental metabolization enzyme in human adults due to the broad substrate specificity which includes, not only a large number of pharmaceutical drugs, but also many xenobiotics (Klein & Zanger, 2013). Although the effect of SNP rs2740574 is still under investigation, it has been suggested that the C allele in this polymorphism increases *CYP3A4* expression due to higher transcriptional activity (Basheer & Kerem, 2015; Klein & Zanger, 2013). Our results show that only one proband was a carrier of the C allele. The proband is heterozygous, and therefore may be unable to benefit from the higher enzymatic activity conferred by this allele. This could lead to an increased clearance of substrates metabolized by *CYP3A4.* In relation to *CYP2D6*, given the low frequency of both *CYP2D6*4* and *CYP2D6*6* (0.1959 and 0.0146 in European Non-Finnish, 0.08025 and 0.002039 in African population), none of the probands were carriers of the minor alleles, which are considered null alleles as they do not encode for functional protein products. However, these SNPs are of high importance given that they can result in the inability to eliminate substrates dependent of *CYP2D6* metabolic activity, thus increasing the probability of adverse drug reactions (Zanger, Raimundo, & Eichelbaum, 2004). Similarly, *CYP2C19*2* also results in loss of function caused by the creation of an aberrant splice site, which produces a truncated, nonfunctional protein. The genotype of intermediate metabolizers (IM) consists in the combination of one wildtype allele and one variant allele, such as *CYP2C19*2*, resulting in decreased *CYP2C19* activity (e.g.:*1/*2), while a poor metabolizer (PM)

genotype consists of two loss-of-function alleles resulting in significantly reduced or absent *CYP2C19* activity (e.g.*2/*2) (Scott et al., 2012). We observed that two probands were heterozygous for this polymorphism (*1/*2), thus suggesting that they are intermediate metabolizers (IM). Both individuals were exposed to pharmaceutical drugs. It is important to add that, as there are more loss-of-function polymorphisms in this gene, we cannot conclude with certainty the metabolizer status of these subjects: for example, if one of the CYP2C19*2 carriers has yet another allele that confers low enzymatic activity, he may be a poor metabolizer. However, if these individuals are truly IMs, then they could still efficiently metabolize the pharmaceutical drugs if this was not present in high concentrations.

Moreover, we analyzed relevant polymorphisms in probands whose mothers took a specific drug during the period covered by the ELEAT. Because we did not receive biological samples from all probands, we were only able to test for polymorphisms related to the activity of acetaminophen and oxytocin. Results from probands who were tested for SNPs affecting the effect of acetaminophen, the most frequently taken medication among mothers, indicated that: out of the three probands tested, one was heterozygous in rs1042640 of the *UGT1A* gene, another was heterozygous in rs1805034 of *TNFRSF11A*, and finally, one was heterozygous of both polymorphisms. However, none carried the alternative allele in rs2228246 of gene *PLCG1*. Homozygous genotype for the minor allele (C) of the *UGT1A* SNP has been associated to an increased risk of liver failure due to unintentional acetaminophen overdose (Court et al., 2013). Meanwhile, due to their involvement in the activation of mast cells, both *TNFRSF11A* and *PLCG1* genes were studied in relation to allergic reactions leading to angioedema and urticaria in patients treated with a number of medications including acetaminophen. In this study, only rs1805034 of *TNFRSF11A* was found in higher frequency among the group that suffered from these allergic reactions compared to control groups, while the frequency of rs2228246 in *PLCG1* was lower in the case group compared to control (Ayusoa et al., 2015). These results are comparable to ours since probands carried the alternative allele of rs1805034 (T) but none carried allele G in rs2228246. None of these SNPs have been directly linked to ASD. However, they are still of some importance as the pathways in which they affect drug activity may indirectly increase ASD risk: the SNP in UGTA affects the liver, which is a vital organ involved in the metabolization of various xenobiotics in the body, and SNPs in *TNFRSF11A* and *PLCG1* are both involved in the abnormal activation of mast cells, which seem to play a key role in inflammation and in the disruption of the blood brain barrier (Polyzoidis, Koletsa, Panagiotidou, Ashkan, & Theoharides, 2015), and thus could be related to ASD risk. Regarding *OXTR*, rs2268498 has been associated with autistic traits in various cultural groups (Montag 2017). Genotyping results revealed that 1 proband was heterozygous (TC), while 2 were homozygous for minor allele (CC). While T allele has been associated with lower *OXTR* expression, which is thought to reflect higher oxytocin sensitivity, the C allele has been associated with higher ASD scores in Autism -Spectrum Quotient test (Montag 2017). Following this notion, it can be presumed that allele C confers higher expression, thus resulting in reduced oxytocin sensitivity, which plays an important role on social cognition. Future studies should screen for this polymorphism in all case patients.

Considering these results, we can conclude that probands were carriers of polymorphisms that may influence the correct processing of certain environmental factors studied by the ELEAT, thus making them more susceptible than the general population. For the ones that were exposed to such xenobiotics during critical developmental periods, this could have played a role in ASD onset. It is important to note that sole exposure to such environmental factors is not sufficient to set in motion the mechanisms that lead to neurodevelopmental problems. It is however the combination of exposure and genetic susceptibility to such factors that may lead to heavier consequences.

This pilot study is an important step for future studies that intend to use the ELEAT to identify environmental risk factors for ASD. The addition of genetic factors has proven to be complementary to the questionnaire and therefore allow for more meaningful results. This combination helps reach more solid conclusions considering the fact that it provides a biological explanation to why the potential factors found by the ELEAT may be associated with ASD risk. As everyone is generally exposed to some levels of these chemicals, genetic factors play a crucial role and cannot be ruled out. Therefore, we believe that the proband's genotype is of crucial importance: knowing if the infant is particularly sensitive to some of the chemicals they were exposed to is essential when considering cause and effect of environmental factors. Consequently, it is important for future studies to not only interpret ELEAT group results, but to also study each case individually and investigate the exposure level of each child to the xenobiotics they may be unable to efficiently metabolize. This information can only be obtained by genotyping each proband, and searching for rare and common variants that are involved in the processing of the xenobiotics studied by this questionnaire.

Nowadays, researchers suggest that ASD is caused by an accumulation of genetic and environmental factors that push the individual to reach a certain threshold, which results in the onset of the pathology (Eapen, 2011; Chaste & Leboyer, 2012). Therefore, ELEAT's ability to measure multiple factors simultaneously gives it an advantage over studies that measure single exposures. It is believed that common variants, like the polymorphisms analyzed in this pilot study, are distributed continually in the general population but can result in a broad range of ASD phenotypes when they accumulate to reach a certain threshold (Eapen, 2011). Further investigation of the contribution of environmental factors through additive or multiplicative effect has been called for (Chaste & Leboyer, 2012), and, as demonstrated in this pilot study, the ELEAT has the potential to explore this area. It is important to note that the tool aims simply to gather information about environmental exposure and is not to be used as a diagnosis instrument.

Taking into account that this is a standardized tool, the ELEAT has the potential of being applied in different countries and gather a large amount of data that could be analyzed together and lead to meaningful findings. It has been noted that the translation of instruments such as the ELEAT may present some cross-cultural problems, thus compromising its validity. However, in this pilot study, these are minimized by the fact that there is not a large discrepancy between the lifestyle and habits of British vs Portuguese mothers. Both have similar access to hospitals, consultations, dietary supplements, baby formula, usage of baby-bottles, etc., which are some of the areas in which the questionnaire focuses on. In terms of comprehensiveness, the Portuguese language is very uniform in the Central Region, thus diminishing the chances of misinterpretation of the questions between the mothers due to different regional terms. In order to ensure that the mothers understand the questions asked, it is important to offer assistance to fill out the questionnaire, thus erasing any doubts they may have.

The main limitation of this tool is its length: as seen by the results in Module I (Instrument Evaluation), the tool is considered to be extensive. This was one of the principal reasons for the limited sample size of this study. Seeing as the questionnaire is 80 pages long and demands at least 1hour to be filled, many mothers did not return to complete it, despite our best efforts. A revision of the questionnaire is recommended, so that a shorter and more direct version could be created. In addition to this, the questions have proven themselves complex to extract quantitative information from, which is necessary for the creation of a score. Therefore, the creation of a rational to transform qualitative into quantitative results is a crucial step to improve the statistical power of ELEAT conclusions. On the other hand, results also showed that mothers felt that the instructions to answer the tool were clear, and the majority felt sure about their

answers. In addition to this, they also appreciated the possibility of filling it online, and gave very positive feedback regarding this process. Feedback from this module is essential, considering that this tool is still being molded to improve its capacity to assemble accurate data from diverse populations. Due to the use of scientific terms in the questionnaire, it is especially important to offer assistance to participants with lower education in order to guarantee accurate data collection. By tackling this and the length of the questionnaire, it is plausible that the selection of "don't know" will decrease. Seeing as indirect measures may not be as accurate as direct ones, the ability of the ELEAT to measure exposure to exogenous factors could be validated by the analysis of neonatal archived dried blood spots: if xenobiotics in blood test match the exposures recorded in the questionnaire, it would confirm the validity of this tool. The main limitation of this pilot study is the sample size. Having only 20 participants, and 14 valid biological samples made it impossible to draw significant statistical results. However, the purpose of this pilot study was to explore the type of results that can be obtained from the ELEAT questionnaire, how these could be related to genetic variants, and thus applied to study gene-environment interactions. Despite the small sample, we were able to reach our goals and demonstrate that this tool, when combined with biological approach, has the potential to yield significant results that may bring about important breakthroughs in the area of gene-environment interaction studies. Now that it has been shown that ELEAT can be applied, we are expanding its application to other regions of Portugal, such as Lisbon.

6. Conclusion & Future Studies

In conclusion, the addition of a biological approach has proven to be complementary to the ELEAT, and lead to more comprehensive results. This pilot study is an important step for future studies that intend to use the this tool to measure environmental factors related to ASD risk. Follow-up studies should include larger population datasets, depending on the effect size of the interaction between genetic susceptibility and environmental factors. An equal number of typically-developing controls should be included. With a large sample, researchers could also study rare genetic variants, which have been shown to be important in ASD, in addition to common ones. Moreover, both biological parents should also be genotyped to provide a more clear understanding of proband's genotype (to know if a given variant is *de novo* or inherited), and the chemicals they were exposed to depending on the mother's metabolism. Despite the limitations of this pilot study, the application of the ELEAT, as well as the genotyping methodology, have been successfully tested. This should be considered one of the strengths of this work. Indeed, once a pipeline has been tested and approved in a smaller pilot sample, all succeeding work should be faster and more consistent. The long-term goal is to use this knowledge to contribute to the development of preventive strategies for autism by adjusting exposure to potentially harmful environmental factors.

BIBLIOGRAPHY

Altman, R. B. (2007). PharmGKB: A logical home for knowledge relating genotype to drug response phenotype. *Nature Genetics*, *39*(4), 426. https://doi.org/10.1038/ng0407-426

American Psychiatric Association. (2013). Diagnostic and statistical manual of mental disorders (5th ed.). Arlington, VA: American Psychiatric Publishing.

Ayusoa, P., Del Carmen Plaza-Seróna, M., Doña, I., Blanca-López, N., Campo, P., Cornejo-García, J. A., … Canto, G. (2015). Association study of genetic variants in PLA2G4A, PLCG1, LAT, SYK, and TNFRS11A genes in NSAIDs-induced urticaria and/or angioedema patients. *Pharmacogenetics and Genomics*, *25*(12), 618–621. https://doi.org/10.1097/FPC.0000000000000179

Basheer, L., & Kerem, Z. (2015). Interactions between CYP3A4 and Dietary Polyphenols. *Oxidative Medicine and Cellular Longevity*, *2015*. https://doi.org/10.1155/2015/854015

Brambila-Tapia, A. J. L. (2013). MDR1 (ABCB1) polymorphisms: Functional effects and clinical implications. *Revista de Investigacion Clinica*, *65*(5), 445–454.

Bremer, L., Goletzke, J., Wiessner, C., Pagenkemper, M., Gehbauer, C., Becher, H., … Tiegs, G. (2017). Paracetamol Medication During Pregnancy: Insights on Intake Frequencies, Dosages and Effects on Hematopoietic Stem Cell Populations in Cord Blood From a Longitudinal Prospective Pregnancy Cohort. *EBioMedicine*, *26*, 146–151. https://doi.org/10.1016/j.ebiom.2017.10.023

Buyske, S., Williams, T. A., Mars, A. E., Stenroos, E. S., Ming, S. X., Wang, R., … Johnson, W. G. (2006). Analysis of case-parent trios at a locus with a deletion allele: Association of GSTMI with autism. *BMC Genetics*, *7*, 1–9. https://doi.org/10.1186/1471-2156-7-8

Carter, C. J. (2016). The barrier, airway particle clearance, placental and detoxification functions of autism susceptibility genes. *Neurochemistry International*, *99*, 42–51. https://doi.org/10.1016/j.neuint.2016.06.003

Castorina, R., Bradman, A., Sjödin, A., Fenster, L., Jones, R. S., Harley, K. G., … Eskenazi, B. (2011). Determinants of Serum Polybrominated Diphenyl Ether (PBDE) Levels among Pregnant Women in the CHAMACOS Cohort. *Environmental Science and Technology*, *45*(15), 6553–6560. https://doi.org/10.1021/es104295m

Chaste, P., & Leboyer, M. (2012). Autism risk factors: genes, environment, and gene-environment interactions Pauline. *Dialogues in Clinical Neuroscience*, *14*(3), 281–292.

Chen, J., Xin, K., Wei, J., Zhang, K., & Xiao, H. (2016). Lower maternal serum 25(OH) D in first trimester associated with higher autism risk in Chinese offspring. *Journal of Psychosomatic Research*, *89*, 98–101. https://doi.org/10.1016/j.jpsychores.2016.08.013

Cieślińska, A., Kostyra, E., Chwała, B., Moszyńska-Dumara, M., Fiedorowicz, E., Teodorowicz, M., & Savelkoul, H. (2017). Vitamin D Receptor Gene Polymorphisms Associated with Childhood Autism. *Brain Sciences*, *7*(9), 115. https://doi.org/10.3390/brainsci7090115

Cornelis, M. C., Byrne, E. M., Esko, T., Nalls, M. A., Ganna, A., Paynter, N., … Chasman, D. I. (2014). Genome-wide meta-analysis identifies six novel loci associated with habitual coffee consumption. *Molecular Psychiatry*, *20*, 647–656. Retrieved from http://dx.doi.org/10.1038/mp.2014.107

Court, M. H., Freytsis, M., Wang, X., Peter, I., Guillemette, C., Hazarika, S., … Lee, W. M. (2013). The UDP-Glucuronosyltransferase (UGT) 1A Polymorphism c.2042C>G (rs8330) Is Associated with Increased Human Liver Acetaminophen Glucuronidation, Increased UGT1A Exon 5a/5b Splice Variant mRNA Ratio, and Decreased Risk of Unintentional Acetaminophen-Ind. *Journal of Pharmacology and Experimental Therapeutics*, *345*(2), 297–307. https://doi.org/10.1124/jpet.112.202010

Cytochrome p450 (2018). Genetics Home Reference. Retrieved from https://ghr.nlm.nih.gov/primer/genefamily/cytochromep450

D'Amelio, M., Ricci, I., Sacco, R., Liu, X., D'Agruma, L., Muscarella, L. A., … Persico, A. M. (2005). Paraoxonase gene variants are associated with autism in North America, but not in Italy: Possible

regional specificity in gene-environment interactions. *Molecular Psychiatry*, *10*, 1006–1016. https://doi.org/10.1038/sj.mp.4001714
Davis, A. P., Grondin, C. J., Johnson, R. J., Sciaky, D., King, B. L., McMorran, R., … Mattingly, C. J. (2017). The Comparative Toxicogenomics Database: Update 2017. *Nucleic Acids Research*, *45*(D1), D972–D978. https://doi.org/10.1093/nar/gkw838
De-Regil, L., Palacios, C., Lombardo, L., & Pena-Rosas, J. (2016). Vitamin D supplementation for women during pregnancy. *The Cochrane Library*, *1*(1), 1–55. https://doi.org/10.1002/14651858.CD008873.pub2.Vitamin
Diamanti-Kandarakis, E., Bourguignon, J. P., Giudice, L. C., Hauser, R., Prins, G. S., Soto, A. M., … Gore, A. C. (2009). Endocrine-disrupting chemicals: An Endocrine Society scientific statement. *Endocrine Reviews*, *30*(4), 293–342. https://doi.org/10.1210/er.2009-0002
Eapen, V. (2011). Genetic basis of autism: Is there a way forward? *Current Opinion in Psychiatry*, *24*(3), 226–236. https://doi.org/10.1097/YCO.0b013e328345927e
Elsabbagh, M., Divan, G., Koh, Y. J., Kim, Y. S., Kauchali, S., Marcín, C., … Fombonne, E. (2012). Global Prevalence of Autism and Other Pervasive Developmental Disorders. *Autism Research*, *5*(3), 160–179. https://doi.org/10.1002/aur.239
Fei, X., Shen, Y., Liu, N., Li, H., Guo, J. and Wu, Z. (2018). Polymorphisms of vitamin D receptor gene TaqI susceptibility of prostate cancer: a meta-analysis. *OncoTargets and Therapy*, 1033–1045. https://doi.org/10.2147/OTT.S99428
Fiorentino, M., Sapone, A., Senger, S., Camhi, S. S., Kadzielski, S. M., Buie, T. M., … Fasano, A. (2016). Blood-brain barrier and intestinal epithelial barrier alterations in autism spectrum disorders. *Molecular Autism*, *7*(1), 1–17. https://doi.org/10.1186/s13229-016-0110-z
Ganesan, S., Comstock, A. T., & Sajjan, U. S. (2013). Barrier function of airway tract epithelium. *Tissue Barriers*, 1(4), 1–9. https://doi.org/10.4161/tisb.24997
Gardener, H., Spiegelman, D., & Buka, S. L. (2009). Prenatal risk factors for autism: Comprehensive meta-analysis. *British Journal of Psychiatry*, *195*(1), 7–14. https://doi.org/10.1192/bjp.bp.108.051672
Geschwind, D. H., & State, M. W. (2015). Gene hunting in autism spectrum disorder: on the to precision medicine. *Lancet Neurology*, *14*(11), 1109–1120. https://doi.org/10.1016/S1474-4422(15)00044-7
Groschwitz, K. R. and Hogan, S. P. (2009). Intestinal Barrier Function: Molecular Regulation and Disease Pathogenesis. *J Allergy Clin Immunol*, *124*(1), 3–20. https://doi.org/10.1016/j.jaci.2009.05.038
Gude, N. M., Roberts, C. T., Kalionis, B., & King, R. G. (2004). Growth and function of the normal human placenta. *Thrombosis Research*, *114*(5–6), 397–407. https://doi.org/10.1016/j.thromres.2004.06.038
Hallmayer, J., Cleveland, S., Torres, A., Phillips, J., Cohen, B., Torigoe, T., … Risch, N. (2011). Genetic Heritability and Shared Environmental Factors Among Twin Pairs With Autism, *68*(11), 1095–1102. https://doi.org/10.1001/archgenpsychiatry.2011.76
Hanioka, N., Naito, T., & Narimatsu, S. (2008). Human UDP-glucuronosyltransferase isoforms involved in bisphenol A glucuronidation. *Chemosphere*, *74*(1), 33–36. https://doi.org/10.1016/j.chemosphere.2008.09.053
Hanioka, N., Oka, H., Nagaoka, K., Ikushiro, S., & Narimatsu, S. (2011). Effect of UDP-glucuronosyltransferase 2B15 polymorphism on bisphenol A glucuronidation. *Archives of Toxicology*, *85*(11), 1373–1381. https://doi.org/10.1007/s00204-011-0690-5
Iossifov, I., Levy, D., Allen, J., Ye, K., Ronemus, M., Lee, Y., … Wigler, M. (2015). Low load for disruptive mutations in autism genes and their biased transmission. *Proceedings of the National Academy of Sciences*, *112*(41), E5600–E5607. https://doi.org/10.1073/pnas.1516376112
Iqbal, M. M., Sobhan, T., & Ryals, T. (2002). Effects of Commonly Used Benzodiazepines on the Fetus, the Neonate, and the Nursing Infant. *Psychiatric Services*, *53*(1), 39–49. https://doi.org/10.1176/appi.ps.53.1.39
Jahed Khanik, G. (2007). Chemical Contaminants in Milk and Public Health Concerns: A Review.

International Journal of Dairy Science, *2*(2), 104–115. https://doi.org/10.3923/ijds.2007.104.115
James, S. J., Melnyk, S., Jernigan, S., Cleves, M. A., Halsted, C. H., Wong, D. H., … Gaylor, D. W. (2006). Metabolic endophenotype and related genotypes are associated with oxidative stress in children with autism. *American Journal Of Medical Genetics Part B Neuropsychiatric Genetics*, *141B*(8), 947–956. https://doi.org/10.1002/ajmg.b.30366
James, S. J., Melnyk, S., Jernigan, S., Lehman, S., Seidel, L., Gaylor, D. . W., & Cleves, M. A. (2010). A functional polymorphism in the reduced folate carrier gene and DNA hypomethylation in mothers of children with autism. *American Journal Of Medical Genetics Part B Neuropsychiatric Genetics*, *153B*(6), 1209–1220. https://doi.org/10.1002/ajmg.b.31094
Jeong, H. (2010). Altered drug metabolism during pregnancy: Hormonal regulation of drug-metabolizing enzymes. *Expert Opinion on Drug Metabolism & Toxicocology*, *6*(6), 689–699. https://doi.org/10.1517/17425251003677755
Kalkbrenner, A. E., Schmidt, R. J., & Penlesky, A. C. (2014). Environmental Chemical Exposures and Autism Spectrum. *Current Problems in Pediatric and Adolescent Health Care*, *44*(10), 277–318. https://doi.org/10.1016/j.cppeds.2014.06.001
Klein, K., & Zanger, U. M. (2013). Pharmacogenomics of cytochrome P450 3A4: Recent progress toward the "missing heritability" problem. *Frontiers in Genetics*, *4*(12), 1–15. https://doi.org/10.3389/fgene.2013.00012
Koss, C. A., Baras, D. C., Lane, S. D., Aubry, R., Marcus, M., Markowitz, L. E., & Koumans, E. H. (2012). Investigation of metronidazole use during pregnancy and adverse birth outcomes. *Antimicrobial Agents and Chemotherapy*, *56*(9), 4800–4805. https://doi.org/10.1128/AAC.06477-11
Koufaris, C., & Sismani, C. (2015). Modulation of the genome and epigenome of individuals susceptible to autism by environmental risk factors. *International Journal of Molecular Sciences*, *16*(4), 8699–8718. https://doi.org/10.3390/ijms16048699
Kulage, K. M., Smaldone, A. M., & Cohn, E. G. (2014). How will DSM-5 affect autism diagnosis? A systematic literature review and meta-analysis. *Journal of Autism and Developmental Disorders*, *44*(8), 1918–1932. https://doi.org/10.1007/s10803-014-2065-2
Landrum, M. J., Lee, J. M., Riley, G. R., Jang, W., Rubinstein, W. S., Church, D. M., & Maglott, D. R. (2014). ClinVar: Public archive of relationships among sequence variation and human phenotype. *Nucleic Acids Research*, *42*(D1), 980–985. https://doi.org/10.1093/nar/gkt1113
Lek, M., Karczewski, K. J., Minikel, E. V., Samocha, K. E., Banks, E., Fennell, T., … MacArthur, D. G. (2016). Analysis of protein-coding genetic variation in 60,706 humans. *Nature*, *536*(7616), 285–291. https://doi.org/10.1038/nature19057
Lionetto, M. G., Caricato, R., Calisi, A., Giordano, M. E., & Schettino, T. (2013). Acetylcholinesterase as a biomarker in environmental and occupational medicine: new insights and future perspectives. *BioMed Research IInternational*, *2013*(1), 1–8. https://doi.org/10.1155/2013/321213
Liu, X., Solehdin, F., Cohen, I. L., Gonzalez, M. G., Jenkins, E. C., Lewis, M. E. S., & Holden, J. J. A. (2011). Population- and family-based studies associate the MTHFR gene with idiopathic autism in simplex families. *Journal of Autism and Developmental Disorders*, *41*(7), 938–944. https://doi.org/10.1007/s10803-010-1120-x
Lyall, K., Schmidt, R. J., & Hertz-Picciotto, I. (2014). Maternal lifestyle and environmental risk factors for autism spectrum disorders. *International Journal of Epidemiology*, *43*(2), 443–464. https://doi.org/10.1093/ije/dyt282
Mehboob, H., Tahir, I. M., Iqbal, T., Saleem, S., Perveen, S., & Farooqi, A. (2017). Effect of UDP-Glucuronosyltransferase (UGT) 1A Polymorphism (rs8330 and rs10929303) on Glucuronidation Status of Acetaminophen. *Dose-Response*, *15*(3), 1–8. https://doi.org/10.1177/1559325817723731
Miller, D. S., Bauer, B., & Hart, A. M. S. (2008). Modulation of P-glycoprotein at the Blood-Brain Barrier: Opportunities to Improve CNS Pharmacotherapy. *Pharmacol Rev.*, *60*(2), 196–209. https://doi.org/10.1124/pr.107.07109
Modabbernia, A., Velthorst, E., & Reichenberg, A. (2017). Environmental risk factors for autism : an evidence-based review of systematic reviews and meta-analyses, *8*(13), 1–16.

https://doi.org/10.1186/s13229-017-0121-4
Møllgård, K., Dziegielewska, K. M., Holst, C. B., Habgood, M. D., & Saunders, N. R. (2017). Brain barriers and functional interfaces with sequential appearance of ABC efflux transporters during human development. *Scientific Reports*, *7*(1), 1–16. https://doi.org/10.1038/s41598-017-11596-0
Montag, C., Sindermann, C., Melchers, M., Jung, S., Luo, R., Becker, B., . . . Kendrick, K. M. (2017). A functional polymorphism of the OXTR gene is associated with autistic traits in Caucasian and Asian populations. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics,174*(8), 808-816. https://doi.org/10.1002/ajmg.b.32596
Moorthy, B., Chu, C., & Carlin, D. J. (2015). Polycyclic aromatic hydrocarbons: From metabolism to lung cancer. *Toxicological Sciences*, *145*(1), 5–15. https://doi.org/10.1093/toxsci/kfv040
Myllynen, P. K., Pienimäki, P. K., & Vähäkangas, K. H. (2003). Transplacental passage of lamotrigine in a human placental perfusion system in vitro and in maternal and cord blood in vivo. *European Journal of Clinical Pharmacology*, *58*(10), 677–682. https://doi.org/10.1007/s00228-002-0544-4
Oliveira, G., Ataíde, A., Marques, C., Miguel, T. S., Coutinho, A. M., Mota-Vieira, L., … Vicente, A. M. (2007). Epidemiology of autism spectrum disorder in Portugal: prevalence, clinical characterization, and medical conditions. *Developmental Medicine & Child Neurology*, *49*(10), 726–733. https://doi.org/10.1111/j.1469-8749.2007.00726.x
Pacifici, G. (2006). Placental transfer of antibiotics administered to the mother: a review. *International Journal of Clinical Pharmacology and Therapeutics*, *44*(2), 57–63. http://dx.doi.org/10.5414/CPP44057
Polyzoidis, S., Koletsa, T., Panagiotidou, S., Ashkan, K., & Theoharides, T. C. (2015). Mast cells in meningiomas and brain inflammation. *Journal of Neuroinflammation*, *12*(1), 1–8. https://doi.org/10.1186/s12974-015-0388-3
Reale, M., Costantini, E., Di Nicola, M., D'Angelo, C., Franchi, S., D'Aurora, M., … Gatta, V. (2018). Butyrylcholinesterase and Acetylcholinesterase polymorphisms in Multiple Sclerosis patients: Implication in peripheral inflammation. *Scientific Reports*, *8*(1), 1–9. https://doi.org/10.1038/s41598-018-19701-7
Sandin, S., Lichtenstein, P., Kuja-Halkola, R., Hultman, C., Larsson, H., & Reichenberg, A. (2017). The Heritability of Autism Spectrum Disorder Analysis method B. *Jama*, *318*(12), 1182–1184. https://doi.org/10.1001/jama.2017.12141
Schmidt, R. J., Hansen, R. L., Hartiala, J., Allayee, H., Sconberg, J. L., Schmidt, L. C., … Flora, T. (2015). Selected vitamin D metabolic gene variants and risk for autism spectrum disorder in the CHARGE Study. *Early Human Development*, *91*(8), 483–489. https://doi.org/10.1016/j.earlhumdev.2015.05.008
Schmidt, R. J., Tancredi, D. J., Ozonoff, S., Hansen, R. L., Hartiala, J., Allayee, H., … Hertz-Picciotto, I. (2012). Maternal periconceptional folic acid intake and risk of autism spectrum disorders and developmental delay in the CHARGE (CHildhood Autism Risks from Genetics and Environment) case-control study. *American Journal of Clinical Nutrition*, *96*(1), 80–89. https://doi.org/10.3945/ajcn.110.004416
Scott, S. A., Sangkuhl, K., Shuldiner, A. R., Hulot, J.-S., Thorn, C. F., Altman, R. B., & Kleinc, T. E. (2012). PharmGKB summary: very important pharmacogene information for cytochrome P450, family 2, subfamily C, polypeptide 19 Stuart. *Pharmacogenetics and Genomics*, *22*(2), 159–165. https://doi.org/10.1097/FPC.0b013e32834d4962
SFARI Gene. (2018). Simons Foundation. Retrieved from https://www.sfari.org/resource/sfari-gene/
Shao, J., Stapleton, P. L., Lin, Y. S., & Gallagher, E. P. (2007). Cytochrome P450 and Glutathione S-Transferase mRNA Expression in Human Fetal Liver Hematopoietic Stem Cells ABSTRACT : *Pharmacology*, *35*(1), 168–175. https://doi.org/10.1124/dmd.106.012757
Sørensen, H. T., Nielsen, G. L., Christensen, K., Tage-Jensen, U., Ekbom, A., & Baron, J. (2000). Birth outcome following maternal use of metoclopramide. The Euromap study group. *British Journal of Clinical Pharmacology*, *49*(3), 264–268. https://doi.org/10.1046/j.1365-2125.2000.00127.x
Sulem, P., Gudbjartsson, D. F., Geller, F., Prokopenko, I., Feenstra, B., Aben, K. K. H., … Stefansson, K.

(2011). Sequence variants at CYP1A1-CYP1A2 and AHR associate with coffee consumption. *Human Molecular Genetics*, *20*(10), 2071–2077. https://doi.org/10.1093/hmg/ddr086

Tordjman, S., Somogyi, E., Coulon, N., Kermarrec, S., Cohen, D., Bronsard, G., … Xavier, J. (2014). Gene × environment interactions in autism spectrum disorders: Role of epigenetic mechanisms. *Frontiers in Psychiatry*, *5*(53), 1–17. https://doi.org/10.3389/fpsyt.2014.00053

Valenzuela, G. J., Craig, J., Bernhardt, M. D., & Holland, M. L. (1995). Placental passage of the oxytocin antagonist atosiban. *American Journal of Obstetrics and Gynecology*, *172*(4), 1304–1306. https://doi.org/10.1016/0002-9378(95)91497-8

Van Heijst, B. F. C., & Geurts, H. M. (2015). Quality of life in autism across the lifespan: A meta-analysis. *Autism*, *19*(2), 158–167. https://doi.org/10.1177/1362361313517053

Vinkhuyzen, A. A. E., Eyles, D. W., Burne, T. H. J., Blanken, L. M. E., Kruithof, C. J., Verhulst, F., … McGrath, J. J. (2017). Gestational vitamin D deficiency and autism spectrum disorder. *British Journal of Psychiatry Open*, *3*(2), 85–90. https://doi.org/10.1192/bjpo.bp.116.004077

Walker, N., Filis, P., Soffientini, U., Bellingham, M., O'Shaughnessy, P. J., & Fowler, P. A. (2017). Placental transporter localization and expression in the human: The importance of species, sex, and gestational age difference. *Biology of Reproduction*, *96*(4), 733–742. https://doi.org/10.1093/biolre/iox012

Wang, M., Li, K., Zhao, D., & Li, L. (2017). The association between maternal use of folic acid supplements during pregnancy and risk of autism spectrum disorders in children: A meta-analysis. *Molecular Autism*, *8*(1), 4–7. https://doi.org/10.1186/s13229-017-0170-8

Werling, D. M. D. M., & Geschwind, D. H. D. H. (2013). Sex differences in autism spectrum disorders. *Current Opinion in Neurology*, *26*(2), 146–153. https://doi.org/10.1097/WCO.0b013e32835ee548

What Are the Symptoms of Autism? (2018). Autism Speaks. Retrieved from https://www.autismspeaks.org/what-autism/symptoms

Wijnen, P. A. H. M., OP Den Buijsch, R. A. M., Drent, M., Kuipers, P. M. J. C., Neef, C., Bast, A., … Koeg, G. H. (2007). Review article: the prevalence and clinical relevance of cytochrome P450 polymorphisms. *Alimentary Pharmacology & Therapeutics*, *26*(2), 211–219. https://doi.org/10.1111/j.1365-2036.2007.03490.x

Yi, B., Kim, C., Park, M., Han, Y., Park, J. Y., & Yang, M. (2013). Association between Endocrine Disrupting Phenols in Colostrums and Maternal and Infant Health, *International Journal of Endocrinology*, *2013*(1), 1–7. https://doi.org/10.1155/2013/282381

Zanger, U. M., Raimundo, S., & Eichelbaum, M. (2004). Cytochrome P450 2D6: Overview and update on pharmacology, genetics, biochemistry. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *369*(1), 23–37. https://doi.org/10.1007/s00210-003-0832-2

ANNEXES

Attachment S1:

Gene	Pharmaceutical drug	Polymorphism	Level of evidence (PharmGKB)	Type of interaction	Nucleotide change	Frequency in European Non-Finnish	Frequency in Africans
UGT1A4	*Lamotrigine*	rs2011425	Level 2B	other	T > G	0.08772	0.1003
HNF4A		rs2071197	Level 3	efficacy/pk	G > A	0.1080	0.02304
SLC22A1		rs628031	Level 3	dosage/efficacy/pk	A > G	0.5852	0.7284
SCN2A		rs2304016	Level 3	efficacy	A > G	0.001916	0.0002946
ABCG2		rs2231142	Level 3	efficacy/pk	G > T	0.1036	0.02738
SCN1A		rs2298771	Level 3	efficacy	C > T	0.6791	0.8287
UGT1A	*Ben-u-ron / Paracetamol*	rs1042640	Level 3	toxicity	G > C	0.7937	0.8008
PLCG1		rs2228246	Level 3	toxicity	A > G	0.1713	0.1011
TNFRSF11A		rs1805034	Level 3	toxicity	C > T	0.5097	0.6446
PLA2G4A		rs12746200	Level 3	toxicity	A > G	0.09034	0.01651

Attachment S1: From the medications that the interviewed mothers reported to take during the period covered by the ELEAT, only four (Ben-u-ron/Paracetamol, Lamotrigine, Clavamox and Valium) had information regarding interaction with specific variants in PharmGKB or Infarmed website. However, as we were only interested in variants that affected toxicity or efficacy of the drug, according to PharmGKB, Clavamox and Valium were excluded, as the reported interactions were of other type. Of the 4 relevant variants reported for Ben-u-ron/Paracetamol, only three were further analyzed (highlighted in blue). None of the variants associated with Lamotrigine were analyzed, as we did not receive biological samples for the offspring of mothers who took this medication. According to PharmGKB, the three analyzed variants have a level of evidence of 3 (from a scale that goes from 1A-4), which means that the annotations for the variant-drug combinations are based on a single significant (not yet replicated) study or in multiple studies that lack clear evidence of an association.

Attachment S2:
Protocol for DNA extraction from blood

1. Thoroughly homogenize blood collected in EDTA and decant into the polypropylene tube;
2. Add to each tube the volume of TKMX-100 equivalent to the initial blood volume (see attached table), and shake vigorously.
3. Add IGEPAL (25µL per mL of blood, see attached table) and shake by inversion until you reach complete solubilization;
4. Centrifuge at 974 rcf (2200 rpm for centrifuge 1011997) for 10minutes at 18 °C, with acceleration 9 and brake 7;
5. Discard the supernatant by decantation into the waste vial;
6. Add TKM1 to the pellet (1 mL per mL of blood, see attached table) and shake by inversion;
7. Centrifuge at 515 rcf (1600 rpm for centrifuge 1011997) for 10minutes at 18 ° C, with acceleration 9 and brake 7. Discard the supernatant.
8. Repeat steps 6, 7 and 8 once (if necessary repeat once more);
9. Resuspend the pellet with TKM2 (160µL per mL of blood, see table attached). Shake vigorously.
10. Add 10% SDS (10µL per mL of blood, see attached table) and shake vigorously;
11. Incubate in the thermostated bath at 55 ° C for 10 ';
12. After incubation, transfer the contents of the polypropylene tube with a Pasteur pipette to a 2.0 ml polypropylene microtube;
13. Add 5M saturated NaCl (60µL per ml of blood, see attached table) and shake well by inversion;
14. Centrifuge for 20minutes at 16,100 rcf (13,200 rpm for centrifuge 1015704) at 18 ° C;
15. Transfer the supernatant with a Pasteur pipette into a 15mL polypropylene tube, without dragging the proteins;
16. Add absolute ethanol (2.3 mL per 5 mL of blood, see attached table) and homogenize slowly by inversion until the DNA pellet is visible;
17. Remove the DNA with a plastic inoculation loop and wash with 70% Ethanol. Let the loop with the DNA rest vertically for approximately 5minutes ;
18. Place the loop in a 1.5mL conical bottom microtube with 150µL of TE and semi-close the tube cap so that the plastic inoculation loop is broken and remains inside.

Attachment I - Solution volume / blood sample volume

Blood	TKMx100	IGEPAL	TKM1	TKM2	SDS	NaCl	EtOH	TE
2,5 mL	2,5mL	62,5µL	2,5mL	400µL	25µL	150µL	1150µL	150µL
3 mL	3mL	75µL	3mL	480µL	30µL	180µL	1380µL	150µL
4mL	4mL	100µL	4mL	640µL	40µL	240µL	1840µL	200µL
5mL	5mL	125µL	5mL	800µL	50µL	300µL	2300µL	200µL

Attachment S3:
Protocol for DNA extraction from buccal swab samples

1. Transfer the solution to a 2ml round bottom tube
2. Centrifuge at 3000rpm for 10min at room temp.
3. Remove 400ul of the supernatant into a 2ml round bottom tube
4. Add 10ul of proteinase K
5. Mix in vortex
6. Incubate for 20 min at 55 ° C
7. Add 160ul TKM2 + 10ul SDS 10%
8. Incubate for 10 min at 55 ° C
9. Add 140 ul of 5M NaCl and homogenize by inversion
10. Centrifuge at 13200rpm for 20 min
11. Remove supernatant to a 2ml round bottom tube
12. Add 500ul of absolute ethanol and shake gently until you see the DNA fibrils
13. Centrifuge at 13200rpm for 1min
14. Remove the supernatant
15. Add 500ul of ethanol 70%
16. Centrifuge at 13200rpm for 1min
17. Remove the supernatant
18. Leave the tubes open inside the chamber for at least 20 min to dry the DNA pellet.
19. Resuspend the DNA in TE (80ul)
20. Quantify and verify DNA integrity.

Attachment S4: Results from selected ELEAT questions

Module B: Breastfeeding and Child Diet

Questions Regarding Breastfeeding and Child's Diet Before His/Her First Birthday		
1. Was your child ever breastfed?	**80%** Yes **20%** No **0%** Declined **0%** Don't Know	
4. Did you ever feed your child infant formula?	**74%** Yes **26%** No **0%** Declined **0%** Don't Know	
4.c. Formulas can come with different protein bases, e.g., milk, soy, or other. What type(s) of formula did you use? [*Mark all that apply*]	**10/14** Cow's milk-based **1/14** Soy-based **2/14** Other(describe)_____ **0/14** Declined **1/14** Don't Know	
5. If you fed your child with a bottle (formula, breast milk, or other liquids), which type of bottle did you use most often?	**70%** Clear hard plastic (no inserts/liners) **0%** Clear hard plastic (with inserts/liners) **0%** Opaque plastic (not clear) **25%** Glass **0%** Never used a bottle **0%** Declined **5%** Don't Know	Was it BPA-free? [such as Born Free®] **4/14** Yes **1/14** No **1/14** Declined **9/14** Don't Know

Module M: Maternal Conditions / Medical Interventions

27. Once you were in labour, were you given a medicine in your IV called oxytocin to speed the process?	**36%** Yes **36%** No **0%** Declined **29%** Don't Know
28. Did you have an infection in your uterus / womb during labour for which they gave you antibiotics?	**14%** Yes **71%** No **0%** Declined **14%** Don't Know

Questions About Chronic Medical Conditions and Dental Care You May Have Had in the 3 Months Before or During Pregnancy

At any time during the **three months before you became pregnant** or during your pregnancy with the child of interest were you treated for any of the following conditions?		If yes, when did you have the condition?	Did you take / were you given any medications for this condition?	Name of medication	Did you take this medication during pregnancy?
45. Asthma?	**0%** Yes **100%** No **0%** Declined **0%** Don't Know	-- In the 3 months before pregnancy -- During pregnancy -- Declined -- Don't know	-- Yes -- No -- Declined -- Don't Know	_____	-- Yes -- No -- Declined -- Don't Know
46. Thyroid disorder?	**0%** Yes **100%** No **0%** Declined **0%** Don't Know	-- In the 3 months before pregnancy -- During pregnancy -- Declined -- Don't know	-- Yes -- No -- Declined -- Don't Know	_____	-- Yes -- No -- Declined -- Don't Know
47. High blood sugar / diabetes?	**5%** Yes - Type 1 **5%** Yes - Type 2 **79%**No **0%** Declined **11%**Don't Know	-- In the 3 months before pregnancy **2/2** During pregnancy -- Declined -- Don't know	**2/2** Yes **1/2** No -- Declined -- Don't Know	_____	**1/2** Yes -- No -- Declined 1/2 Don't know
48. High blood pressure / hypertension?	**15%** Yes **80%** No **0%** Declined **5%** Don't Know	-- In the 3 months before pregnancy **3/3** During pregnancy -- Declined -- Don't know	**1/3** Yes **1/3** No -- Declined -- Don't Know	_____	**2/3** Yes **1/3** No -- Declined -- Don't know
49. Did you have dental cleanings or other dental procedures?	**30%** Yes **55%** No **0%** Declined **15%**Don't Know	**2/6** In the 3 months before pregnancy **4/6** During pregnancy -- Declined **1/6** Don't know	-- Yes **6/6** No -- Declined -- Don't Know	_____	-- Yes -- No -- Declined -- Don't know

Questions About Medical Conditions or Procedures You May Have Experienced During Your Pregnancy			
At any time during your pregnancy with the child of interest did you have any of the following conditions or procedures?		Did you take / were you given any medication for this condition?	Name of medication:
50. High blood sugar / diabetes? *(Gestational Diabetes)*	**10%** Yes **80%** No **0%** Declined **10%**Don't Know	**1/2** Yes **1/2** No -- Declined -- Don't Know	_____
51. Anaemia / low blood count?	**15%** Yes **80%** No **0%** Declined **5%** Don't Know	**1/3** Yes **1/3** No -- Declined **1/3** Don't Know	_____
52. Depression - diagnosed by a health provider?	-- Yes **100%** No -- Declined -- Don't Know	-- Yes -- No -- Declined -- Don't Know	_____
53. Vaginal bleeding?	-- Yes **100%** No -- Declined -- Don't Know	-- Yes -- No -- Declined -- Don't Know	_____
54. Severe nausea and vomiting / hyperemesis?	**15%** Yes **80%** No **0%** Declined **5%** Don't Know	**2/3** Yes **1/3** No -- Declined -- Don't Know	**Nausefe**
55. Preeclampsia / toxaemia?	**5%** Yes **95%** No **0%** Declined **0%** Don't Know	-- Yes **1/1** No -- Declined -- Don't Know	_____
56. Early / preterm labour?	**5%** Yes **95%** No **0%** Declined **0%** Don't Know	**1/1** Yes -- No -- Declined -- Don't Know	**Atosiban**
57. Smaller than average growth of the baby?	**15%** Yes **75%** No **0%** Declined **10%** Don't Know	-- Yes **3/3** No -- Declined -- Don't Know	_____
58. Any other serious condition? 2 Migraine 1 Epilepsy 1 systemic lupus erythematosus (SLE)	**20%** Yes **80%** No **0%** Declined **0%** Don't Know	**4/4** Yes -- No -- Declined -- Don't Know	**Ben-u-ron** **Primperan** **Paracetamol** **Lamotrigine**

During your pregnancy with the child of interest did you have any of the following conditions?	If yes, when did you have the condition?	Name of procedure used to treat this condition:
61. Was your blood type Rh-negative? *[Women with this condition get a shot called Rho(D) immune globulin/RhoGam in the 3rd trimester and after delivery]*	**5%** Yes **90%** No **0%** Declined **0%** Don't Know	**Got a shot after delivery**

Questions About Infections You May Have Experienced During Your Pregnancy				
At any time during your pregnancy with the child of interest did you have any of the following infections?		If yes, when did you have the infection?	Did you take / were you given any medication for this infection?	Name of medication:
62. Influenza / the flu?	**20%** Yes **55%** No **0%** Declined **25%** Don't Know	**1/4** 1st Trimester **2/4** 2nd Trimester **1/4** 3rd Trimester -- Declined **1/4** Don't Know	**4/4** Yes -- No -- Declined -- Don't Know	**Ovule for vaginal infection** **Clavamox** **Ben-u-ron**
63. Fever?	**5%** Yes **84%** No **0%** Declined **11%** Don't Know	-- 1st Trimester **1/1** 2nd Trimester -- 3rd Trimester -- Declined -- Don't Know	**1/1** Yes -- No -- Declined -- Don't Know	***Be-u-ron***
64. Bladder / kidney / urinary tract infection?	**15%** Yes **80%** No **0%** Declined **5%** Don't Know	-- 1st Trimester **2/3** 2nd Trimester **1/3** 3rd Trimester -- Declined -- Don't Know	**3/3** Yes -- No -- Declined -- Don't Know	**Canesten Ovule**
65. Genital herpes?	**5%** Yes **90%** No **0%** Declined **5%** Don't Know			
65.a. Chronic condition without outbreak		-- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- Declined -- Don't Know	-- Yes -- No -- Declined -- Don't Know	
65.b. Outbreak(s)		-- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- Declined -- Don't Know	-- Yes -- No -- Declined -- Don't Know	
66. Bacterial vaginal infection / bacterial vaginosis / BV?	**6%** Yes **83%** No **0%** Declined **11%** Don't Know	-- 1st Trimester -- 2nd Trimester **1/1** 3rd Trimester -- Declined -- Don't Know	**1/1** Yes -- No -- Declined -- Don't Know	***metronidazole***
67. Yeast vaginal infection?	**15%** Yes **75%** No **0%** Declined **10%** Don't Know	-- 1st Trimester **1/3** 2nd Trimester **1/3** 3rd Trimester -- Declined **1/3** Don't Know	**3/3** Yes -- No -- Declined -- Don't Know	***Canesten***
68. Trichomonas / Trich vaginal infection?	**0%** Yes **89%** No **0%** Declined **11%** Don't Know	-- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- Declined -- Don't Know	-- Yes -- No -- Declined -- Don't Know	

Questions About Other Medication Use in the 3 Months Before Pregnancy, During Your Pregnancy, or While Breastfeeding			
Did you take any of the following medication types – in addition to the ones previously listed?		If yes, when did you take this medication?	Name of medication
71. Prescription medicines for pain, fever, inflammation?	**25%** Yes **65%** No **0%** Declined **10%** Don't Know	**1/5** In the 3 months before pregnancy **5/5** During Pregnancy **3/5** While Breastfeeding -- Declined -- Don't Know	_____
72. Non-prescription medicines for pain, fever, inflammation?	**0%** Yes **95%** No **0%** Declined **5%** Don't Know	-- In the 3 months before pregnancy -- During Pregnancy -- While Breastfeeding -- Declined -- Don't Know	_____
73. Migraine medication?	**10%** Yes **90%** No **0%** Declined **0%** Don't Know	-- In the 3 months before pregnancy **2/2** During Pregnancy **1/2** While Breastfeeding -- Declined -- Don't Know	**Ben-u-ron** **Primperan**
74. Muscle relaxants?	**5%** Yes **95%** No **0%** Declined **0%** Don't Know	**1/1** In the 3 months before pregnancy -- During Pregnancy -- While Breastfeeding -- Declined -- Don't Know	_____
75. Allergy medication?	**5%** Yes **95%** No **0%** Declined **0%** Don't Know	-- In the 3 months before pregnancy **1/1** During Pregnancy **1/1** While Breastfeeding -- Declined -- Don't Know	_____
76. Acne Medication?	**0%** Yes **100%** No **0%** Declined **0%** Don't Know	-- In the 3 months before pregnancy -- During Pregnancy -- While Breastfeeding -- Declined -- Don't Know	_____
77. Sedatives / sleep aids?	**0%** Yes **100%** No **0%** Declined **0%** Don't Know	-- In the 3 months before pregnancy -- During Pregnancy -- While Breastfeeding -- Declined -- Don't Know	_____
78. Female hormone medication? (for birth control, to get pregnant, other reasons)	**20%** Yes **75%** No **0%** Declined **5%** Don't Know		
78.a. For birth control	**1/4** In the 3 months before pregnancy -- During Pregnancy **1/4** While Breastfeeding -- Declined **2/4** Don't Know	**Cerazette** **Harmonet**	
78.b. To get pregnant	**2/4** In the 3 months before pregnancy -- During Pregnancy **1/4** While Breastfeeding -- Declined **1/4** Don't Know	**Cerazette**	

79. Medicine for mental health disorder other than depression?	**5%** Yes **95%** No **0%** Declined **0%** Don't Know	-- In the 3 months before pregnancy **1/1** During Pregnancy -- While Breastfeeding -- Declined -- Don't Know	**Valium**
80. Seizure medication?	**5%** Yes **15%** No **0%** Declined **0%** Don't Know	**1/1** In the 3 months before pregnancy **1/1** During Pregnancy **1/1** While Breastfeeding -- Declined -- Don't Know	**Lamotrigine**
81. Cough medication?	**5%** Yes **95%** No **0%** Declined **0%** Don't Know	-- In the 3 months before pregnancy **1/1** During Pregnancy -- While Breastfeeding -- Declined -- Don't Know	
82. Other medication?	**21%** Yes **79%** No **0%** Declined **0%** Don't Know	-- In the 3 months before pregnancy **4/4** During Pregnancy -- While Breastfeeding -- Declined -- Don't Know	**Magnesium** **Iron** **Calcium** **Kompensann S** **Antibiotic**

Module D: Maternal Diet

Questions about your diet before pregnancy, during pregnancy and while breastfeeding.	
Did you consume any bottled water?	**65%** Yes **15%** No **0%** Declined **20%** Don't Know

***During pregnancy*, about how often did you usually eat or drink each of the following foods?**												
	Never	Less than 1 time per Month	1-3 times per Month	1-2 times per Week	3-4 times per Week	5-6 times per Week	1 time per Day	2 times per Day	3 times per Day	4 times per Day	5 times or more per Day	Don't know
5. Cheese	Never **10%**	<1/mo **10%**	1-3/mo **5%**	1-2/wk **25%**	3-4/wk **15%**	5-6/wk **10%**	1/d **10%**	2/d **0%**	3/d **0%**	4/d **0%**	5+/d **0%**	**15%**
25. Eggs	Never **10%**	<1/mo **10%**	1-3/mo **20%**	1-2/wk **30%**	3-4/wk **15%**	5-6/wk **0%**	1/d **10%**	2/d **0%**	3/d **0%**	4/d **0%**	5+/d **0%**	**5%**
26. Fish	Never **5%**	<1/mo **5%**	1-3/mo **0%**	1-2/wk **35%**	3-4/wk **25%**	5-6/wk **10%**	1/d **15%**	2/d **0%**	3/d **0%**	4/d **0%**	5+/d **0%**	**5%**
26.a. What type[s] of fish did you eat most often?	**10%** Haddock **0%** Fresh Tuna **20%** Canned Tuna **30%** Codfish **0%** Sea Bream **0%** *Cação* **40%** Horse Mackerel **10%** Mackerel			**5%** Wreakfish **35%** Gilthead bream **10%** Soleed **10%** Snapper **15%**Scabbard **0%** Catfish **35%** Hake **25%** Bass		**15%** Fresh Salmon **0%** Canned Salmon **0%** Red Mullet **5%** Sole **10%** Trout **10%** Other Fish: **Sardine, Redfish, Maruca** **0%** Declined **5%** Don't Know						
29. Red Meat	Never **0%**	<1/mo **10%**	1-3/mo **25%**	1-2/wk **30%**	3-4/wk **20%**	5-6/wk **5%**	1/d **5%**	2/d **0%**	3/d **0%**	4/d **0%**	5+/d **0%**	**5%**

Module S: Supplements

Questions About Nutrient or Herbal Supplements You May Have Taken			
1. Vitamin or supplement containing folic acid		**65%** Yes **25%** No **0%** Declined **10%** Don't Know	
1.a. When?	Before Pregnancy? **4/13** Yes **9/13** No -- Declined -- Don't Know	During Pregnancy? **8/13** Yes [*Mark when*] **8/13** 1st Month **7/13** 2nd Month **8/13** 3rd Month **4/13** 2nd Trimester **4/13** 3rd Trimester **2/13** Unsure When -- No -- Declined -- Don't Know	While Breastfeeding? **1/13** Did Not Breastfeed **2/13** Yes **9/13** No -- Declined -- Don't Know
2. Prenatal vitamins		**45%** Yes **50%** No **0%** Declined **5%** Don't Know	
2.a. When?	Before Pregnancy? **1/9** Yes **6/9** No **3/9** Declined -- Don't Know	During Pregnancy? **7/9** Yes [*Mark when*] **5/9** 1st Month **4/9** 2nd Month **4/9** 3rd Month **3/9** 2nd Trimester **4/9** 3rd Trimester -- Unsure When -- No -- Declined **3/9** Don't Know	While Breastfeeding? **2/9** Did Not Breastfeed -- Yes **6/9** No -- Declined **2/9** Don't Know
2.c. Did the prenatal vitamin you took contain iron?		**6/9** Yes **2/9** No -- Declined -- Don't Know	
3. Other Multivitamins		**21%** Yes **68%** No **0%** Declined **11%** Don't Know	
3.a. When?	Before Pregnancy? -- Yes **4/4** No -- Declined -- Don't Know	During Pregnancy? **4/4** Yes [*Mark when*] **2/4** 1st Trimester **2/4** 2nd Trimester **2/4** 3rd Trimester -- Unsure When -- No -- Declined -- Don't Know	While Breastfeeding? -- Did Not Breastfeed **1/4** Yes **2/4** No -- Declined **1/4** Don't Know
3.c. Did the multivitamin you took contain iron?		**5/4** Yes -- No -- Declined -- Don't Know	

Did you take any of the following single vitamin, mineral, or herbal supplements?		**a.** If yes, when?	**b.** How often?
4. Iron?	**70%** Yes **15%** No **0%** Declined **15%** Don't Know	**3/14** In 3 Months Before Pregnancy **3/14** 1st Trimester **5/14** 2nd Trimester **5/14** 3rd Trimester **4/14** During Pregnancy, Unsure When **1/14** While Breastfeeding (Mos 1-6) -- Declined **2/14** Don't Know	**11/14** Daily or More -- 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined **3/14** Don't Know
5. Folic Acid?	**80%** Yes **15%** No **0%** Declined **5%** Don't Know	**5/16** In 3 Months Before Pregnancy **7/16** 1st Trimester **4/16** 2nd Trimester **4/16** 3rd Trimester **4/16** During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) -- Declined **3/16** Don't Know	**13/16** Daily or More -- 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined **3/14** Don't Know
6. B complexes (Stress Tabs)?	**15%** Yes **75%** No **0%** Declined **10%** Don't Know	-- In 3 Months Before Pregnancy **1/3** 1st Trimester **2/3** 2nd Trimester **1/3** 3rd Trimester -- During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) -- Declined **2/3** Don't Know	**1/3** Daily or More **1/3** 4-6 Days Per Week **1/3** 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined **2/3** Don't Know
7. Vitamin B12?	**5%** Yes **75%** No **0%** Declined **20%** Don't Know	-- In 3 Months Before Pregnancy -- 1st Trimester -- 2nd Trimester -- 3rd Trimester **1/1** During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) -- Declined **1/1** Don't Know	**1/1** Daily or More -- 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined **1/1** Don't Know
8. Vitamin B6?	**0%** Yes **80%** No **0%** Declined **20%** Don't Know	-- In 3 Months Before Pregnancy -- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) -- Declined -- Don't Know	-- Daily or More -- 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined -- Don't Know
9. Vitamin A or Retinol?	**0%** Yes **89%** No **0%** Declined **11%** Don't Know	-- In 3 Months Before Pregnancy -- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) -- Declined -- Don't Know	-- Daily or More -- 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined -- Don't Know

10. Beta Carotene?	**0%** Yes **90%** No **0%** Declined **10%** Don't Know	-- In 3 Months Before Pregnancy -- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) -- Declined -- Don't Know	-- Daily or More -- 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined -- Don't Know
11. Vitamin E?	**0%** Yes **89%** No **0%** Declined **11%** Don't Know	-- In 3 Months Before Pregnancy -- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) -- Declined -- Don't Know	-- Daily or More -- 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined -- Don't Know
12. Calcium?	**32%** Yes **53%** No **0%** Declined **16%** Don't Know	**1/6** In 3 Months Before Pregnancy **1/6** 1st Trimester **2/6** 2nd Trimester **2/6** 3rd Trimester **2/6** During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) **1/6** Declined **2/6** Don't Know	**4/6** Daily or More -- 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined **3/6** Don't Know
13. Niacin?	**0%** Yes **89%** No **0%** Declined **11%** Don't Know	-- In 3 Months Before Pregnancy -- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) -- Declined -- Don't Know	-- Daily or More -- 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined -- Don't Know
14. Selenium?	**0%** Yes **89%** No **0%** Declined **11%** Don't Know	-- In 3 Months Before Pregnancy -- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) -- Declined -- Don't Know	-- Daily or More -- 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined -- Don't Know
15. Zinc?	**0%** Yes **89%** No **0%** Declined **11%** Don't Know	-- In 3 Months Before Pregnancy -- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) -- Declined -- Don't Know	-- Daily or More -- 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined -- Don't Know

16. Fish Oil or Omega 3 Fatty Acids?	**0%** Yes **89%** No **0%** Declined **11%** Don't Know	-- In 3 Months Before Pregnancy -- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) -- Declined -- Don't Know	-- Daily or More -- 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined -- Don't Know
17. Flaxseed?	**5%** Yes **89%** No **0%** Declined **5%** Don't Know	**1/1** In 3 Months Before Pregnancy **1/1** 1st Trimester **1/1** 2nd Trimester **1/1** 3rd Trimester **1/1** During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) -- Declined **1/1** Don't Know	-- Daily or More **1/1** 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined **1/1** Don't Know

18. Ginko biloba?	0% Yes 95% No 0% Declined 5% Don't Know	-- In 3 Months Before Pregnancy -- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) -- Declined -- Don't Know	-- Daily or More -- 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined -- Don't Know
19. Ginseng?	0% Yes 95% No 0% Declined 5% Don't Know	-- In 3 Months Before Pregnancy -- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) -- Declined -- Don't Know	-- Daily or More -- 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined -- Don't Know
20. St. John's Wort?	0% Yes 95% No 0% Declined 5% Don't Know	-- In 3 Months Before Pregnancy -- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) -- Declined -- Don't Know	-- Daily or More -- 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined -- Don't Know
21. Co-enzyme Q-10?	0% Yes 89% No 0% Declined 11% Don't Know	-- In 3 Months Before Pregnancy -- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) -- Declined -- Don't Know	-- Daily or More -- 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined -- Don't Know
22. Probiotics?	0% Yes 89% No 0% Declined 11% Don't Know	-- In 3 Months Before Pregnancy -- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) -- Declined -- Don't Know	-- Daily or More -- 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined -- Don't Know
23. Others? Describe: **<u>Magnesium</u>**	5% Yes 79% No 0% Declined 11% Don't Know	-- In 3 Months Before Pregnancy -- 1st Trimester 1/1 2nd Trimester 1/1 3rd Trimester -- During Pregnancy, Unsure When -- While Breastfeeding (Mos 1-6) -- Declined 1/1 Don't Know	1/1 Daily or More -- 4-6 Days Per Week -- 1-3 Days Per Week -- 1-4 Days Per Month -- Less Than Once per Month -- Declined 1/1 Don't Know

Module L: Lifestyle

Questions About Lifestyle and Substance Use			
3. During pregnancy, about how many hours per week did you spend outside, on average?	The average for 9 answers is: **6.2** hours per week		
3.a. How many of those hours were during sunny periods?	The average for 9 answers is: **3.6** hours per week		
Did you ever consume/use any of the following substances in the 3 months before pregnancy, during your pregnancy, or during the first 6 months of breastfeeding (or while feeding your child breast milk)?			
5. Caffeinated soda?	**11%** Yes **74%** No **0%** Declined **16%** Don't Know		
5.a. When?	Before Pregnancy? **2/2** Yes -- No -- Declined -- Don't Know	During Pregnancy? **1/2** Yes [*Mark when*] -- 1st Trimester -- 2nd Trimester -- 3rd Trimester 1/2 Unsure When -- No -- Declined **1/2** Don't Know	While Breastfeeding? -- Did Not Breastfeed -- Yes **1/2** No -- Declined **1/2** Don't Know
6. Caffeinated tea?	**15%** Yes **75%** No **0%** Declined **10%** Don't Know		
6.a. When?	Before Pregnancy? **3/3** Yes -- No -- Declined -- Don't Know	During Pregnancy? **3/3** Yes [*Mark when*] **1/3** 1st Trimester **1/3** 2nd Trimester **1/3** 3rd Trimester **1/3** Unsure When -- No -- Declined -- Don't Know	While Breastfeeding? **2/3** Did Not Breastfeed **1/3** Yes -- No -- Declined -- Don't Know
7. Caffeinated coffee?	**60%** Yes **40%** No **0%** Declined **0%** Don't Know		
7.a. When?	Before Pregnancy? **12/12** Yes -- No -- Declined -- Don't Know	During Pregnancy? **8/12** Yes [*Mark when*] **6/12** 1st Trimester **6/12** 2nd Trimester **6/12** 3rd Trimester **1/12** Unsure When **2/12** No -- Declined **1/12** Don't Know	While Breastfeeding? **2/12** Did Not Breastfeed **6/12** Yes **3/16** No -- Declined -- Don't Know
8. Caffeinated espresso drinks?	**35%** Yes **50%** No **0%** Declined **15%** Don't Know		

8.a. When?	Before Pregnancy? **7/7** Yes -- No -- Declined -- Don't Know	During Pregnancy? **4/7** Yes [*Mark when*] **2/7** 1st Trimester **1/7** 2nd Trimester **2/7** 3rd Trimester **2/7** Unsure When -- No -- Declined -- Don't Know	While Breastfeeding? **1/7** Did Not Breastfeed **3/7** Yes **2/7** No -- Declined -- Don't Know
9. Caffeinated energy drinks?	**5%** Yes **95%** No **0%** Declined **0%** Don't Know		
9.a. When?	Before Pregnancy? **1/1** Yes -- No -- Declined -- Don't Know	During Pregnancy? **1/1** Yes [*Mark when*] **1/1** 1st Trimester **1/1** 2nd Trimester **1/1** 3rd Trimester -- Unsure When -- No -- Declined -- Don't Know	While Breastfeeding? -- Did Not Breastfeed **1/1** Yes -- No -- Declined -- Don't Know
10. Cigarettes?	**5%** Yes **95%** No **0%** Declined **0%** Don't Know		
10.a. When?	Before Pregnancy? **1/1** Yes -- No -- Declined -- Don't Know	During Pregnancy? -- Yes [*Mark when*] -- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- Unsure When **1/1** No -- Declined -- Don't Know	While Breastfeeding? -- Did Not Breastfeed -- Yes **1/1** No -- Declined -- Don't Know
11. E-cigarettes or ENDD (Electronic Nicotine Delivery Device)?	**0%** Yes **100%** No **0%** Decline **0%** Don't Know		
12. Other tobacco or nicotine products?	**0%** Yes **100%** No **0%** Decline **0%** Don't Know		
13. Alcohol (any type)?	**0%** Yes **100%** No **0%** Decline **0%** Don't Know		
14. Marijuana / Hashish?	**0%** Yes **100%** No **0%** Decline **0%** Don't Know		
15. Other recreational, illicit or street drugs?	**0%** Yes **100%** No **0%** Decline **0%** Don't Know		

<table>
<tr><td colspan="2">16. Did the biological father smoke cigarettes during the three months before conception?</td><td colspan="2">30% Yes
70% No
0% Declined
0% Don’t Know</td></tr>
<tr><td colspan="2">17. Did you live with anyone before, during or after your pregnancy who smoked cigarettes?</td><td colspan="2">47% Yes
53% No
0% Declined
0% Don’t Know</td></tr>
<tr><td>17.a. If yes, when did you live with the person who smoked cigarettes?</td><td>Before Pregnancy?
9/9 Yes
-- No
-- Declined
-- Don’t Know</td><td>During Pregnancy?
6/9 Yes [Mark when]
5/9 1st Trimester
5/9 2nd Trimester
5/9 3rd Trimester
-- Unsure When
1/9 No
-- Declined
-- Don’t Know</td><td>First Year of Life?
7/9 Yes
1/9 No
-- Declined
-- Don’t Know</td></tr>
<tr><td>17.b. Were cigarettes smoked inside your home?</td><td>Before Pregnancy?
2/9 Yes
6/9 No
-- Declined
-- Don’t Know</td><td>During Pregnancy?
2/9 Yes [Mark when]
1/9 1st Trimester
1/9 2nd Trimester
1/9 3rd Trimester
-- Unsure When
6/9 No
-- Declined
-- Don’t Know</td><td>First Year of Life?
3/9 Yes
6/9 No
-- Declined
-- Don’t Know</td></tr>
<tr><td colspan="2">18. Did anyone who lived in your home smoke any other products anywhere inside your home?</td><td colspan="2">0% Yes
100% No
0% Decline
0% Don’t Know</td></tr>
</table>

Module H: Home Environment

2. These answers relate to: **89%** The home I lived in during all of pregnancy and my child's first year of life **11%** The home I lived in for the longest period of time during pregnancy and my child's first year **0%** The home I lived in for the second longest period of time during pregnancy and my child's first year	
5. What was the type of home?	**25%** Detached House **10%** Semi-detached House **5%** Terraced House **35%** Low rise flat (1-3 storey building **10%** High rise flat (4+ storey building) **0%** Mobile home/ Caravan **10%** Social service housing (social) **5%** Other [Specify:] __lived in a car____ **0%** Declined **0%** Don't Know
8. Was there an enclosed garage attached to this home?	**58%** Yes **42%** No **0%** Declined **0%** Don't Know
9. Were cars, vans, trucks, or other motor vehicles parked in this attached garage? [*include quad bikes, motorcycles*]	**91%** Yes **9%** No **0%** Declined **0%** Don't Know
10. Were any petrol powered devices stored in any room, basement, or attached garage in this home?	**11%** Yes **89%** No **0%** Declined **0%** Don't Know
11. Did your drinking water come from your own private well or were you on a public water supply at this home?	**16%** Private well **79%** Public water supply **0%** Declined **5%** Don't know
11.a. Did you ever have your private well water tested for lead or other contaminants?	**0%** Yes **40%** No **0%** Declined **60%** Don't Know
11.b. What were the results?	-- No problems were ever reported It contained [*mark all that apply*] -- Lead -- Nitrates/nitrites -- Pesticides -- Arsenic -- Copper -- *Giardia,Legionella, Shigella, Campybacter, Salmonella, Cryptosporidium,* or *E. coli* or other bacteria -- Norovirus -- Hepatitis A -- Other [Specify]: _____ -- Declined -- Don't know
12. Did you drink your tap water?	**45%** Yes **55%** No **0%** Declined **0%** Don't Know

12.a. Did you filter the tap water in any way?	**1/9** Yes, using a pitcher [e.g. Brita] **0/9** Yes, using a system in the refrigerator **2/9** Yes, using a system in the sink/faucet **0/9** Yes, other [Specify:] _____ **6/9** No **0/9** Declined **1/9** Don't Know
13. What was the main heating source in the home?	**0%** Gas-Heated forced air (vents) **15%** Electric-heated forced air (vents) **25%** Oil space heater **20%** Radiators (steam or hot water) **35%** Gas stove/fireplace/wall furnace **0%** Fireplace **0%** Kerosene space heater **0%** Ceramic heater **10%** Electric space heater **0%** Other [Specify:] _____ **20%** No source of heat **0%** Declined **0%** Don't know
14. Was air conditioning (refrigeration) used to cool this home?	**16%** Yes **84%** No **0%** Declined **0%** Don't Know
14.a. What type?	-- Central unit/units **2/3** Window or wall unit/units **1/3** Portable unit/units -- Evaporative Cooler -- Other. Specify: _____ -- Declined -- Don't know
15. Did this home have peeling paint on the inside?	**11%** Yes **89%** No **0%** Declined **0%** Don't Know
16. Did this home have either linoleum or vinyl flooring in any of the rooms?	**0%** Yes **95%** No **0%** Declined **5%** Don't Know
16.a. How many rooms had vinyl or linoleum flooring?	--- Number
17. What kind of stove did this home have?	**85%** Gas **15%** Electric **0%** Other [Specify:] _____ **0%** Declined **0%** Don't know

18. Was there a fume hood/fan over the stove?	**79%** Yes **21%** No **0%** Declined **0%** Don't Know
18.a. How often did you use it?	**87%** All/most of the time **13%** About half of the time **0%** Rarely/never **0%** Declined **0%** Don't Know
19. Did this home have any mold or mildew on walls or other surfaces other than in the shower or bathtub?	**32%** Yes **63%** No **0%** Declined **5%** Don't Know
20. Did this home have any water damage?	**26%** Yes **74%** No **0%** Declined **0%** Don't Know
21. Was this home within ~400 meters of an agricultural field or golf course?	**37%** Yes **63%** No **0%** Declined **0%** Don't Know
22. Was this home within ~400 meters of an landfill?	**0%** Yes **95%** No **0%** Declined **5%** Don't Know
23. Was this home within ~400 meters of an a busy road/highway?	**53%** Yes **47%** No **0%** Declined **0%** Don't Know

Module E: Environment

Questions about furnishings, renovations and consumer product use

The following questions are relevant for the time period between three months prior to becoming pregnant to the end of the first year after birth. If the response is yes, please tell us time period the event occurred – mark all that apply.

1. How many pieces of furniture with cushions, such as sofas or chairs with cushions, were in your home while you were pregnant?	The average for 8 answers is: **5.6** pieces of furniture		
3. From three months prior to when you became pregnant through the end of the first year after birth, were there any renovations to your home, such as adding a room, putting up or taking down a wall, replacing windows, or refinishing floors?	**21%** Yes **74%** No **0%** Declined **5%** Don't Know		
3.a. When?	Before Pregnancy? **3/4** Yes **1/4** No -- Declined -- Don't Know	During Pregnancy? **1/4** Yes [*Mark when*] -- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- Unsure When **3/4** No -- Declined -- Don't Know	First Year? **1/4** 0-6 Months **1/4** 6-12 Months **1/4** No -- Declined **1/4** Don't Know
4. From three months prior to when you became pregnant through the end of the first year after birth, were any walls, ceilings, or furniture inside of this home freshly painted / varnished?	**35%** Yes **50%** No **0%** Declined **15%** Don't Know		
4.a. When?	Before Pregnancy? **4/7** Yes **2/7** No -- Declined **1/7** Don't Know	During Pregnancy? **2/7** Yes [*Mark when*] -- 1st Trimester -- 2nd Trimester **2/7** 3rd Trimester **1/7** Unsure When **2/7** No -- Declined **1/7** Don't Know	First Year? **1/7** 0-6 Months **2/7** 6-12 Months **4/7** No -- Declined **1/7** Don't Know
5. From three months prior to when you got pregnant through the end of the first year after birth were new carpets or rugs installed?	**60%** Yes **25%** No **0%** Declined **15%** Don't Know		
5.a. When?	Before Pregnancy? **9/12** Yes **1/12** No -- Declined **2/12** Don't Know	During Pregnancy? **3/12** Yes [*Mark when*] **5/12** 1st Trimester **4/12** 2nd Trimester **4/12** 3rd Trimester **2/12** Unsure When **2/12** No -- Declined **2/12** Don't Know	First Year? **5/12** 0-6 Months **5/12** 6-12 Months -- No -- Declined **4/12** Don't Know

6. Was any pesticide fogger / fumigator used **inside** your home from three months prior to becoming pregnant through the end of the child's first year after birth?	**5%** Yes **80%** No **0%** Declined **15%** Don't Know		
6.a. When	Before Pregnancy? **1/1** Yes -- No -- Declined -- Don't Know	During Pregnancy? -- Yes [*Mark when*] -- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- Unsure When **1/1** No -- Declined -- Don't Know	First Year? -- 0-6 Months -- 6-12 Months **1/1** No -- Declined -- Don't Know
6.b. Which pests were you treating?	**1/1**Cockroaches -- Bees/Wasps **1/1**Ants -- Flies **2/1**Mosquitos -- Termites -- Fleas -- Moths -- Spiders -- Snails -- Rodents -- Declined -- Don't Know	-- Cockroaches -- Bees/Wasps -- Ants -- Flies -- Mosquitos -- Termites -- Fleas -- Moths -- Spiders -- Snails -- Rodents -- Declined -- Don't Know	-- Cockroaches -- Bees/Wasps -- Ants -- Flies -- Mosquitos -- Termites -- Fleas -- Moths -- Spiders -- Snails -- Rodents -- Declined -- Don't Know
7. Was any pesticide spray used **inside** your home from three months prior to becoming pregnant through the end of the child's first year after birth?	**16%** Yes **74%** No **0%** Declined **11%**Don't Know		
7.a. When?	Before Pregnancy? **2/3** Yes -- No -- Declined -- Don't Know	During Pregnancy? -- Yes [*Mark when*] -- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- Unsure When **1/3** No -- Declined -- Don't Know	First Year? -- 0-6 Months -- 6-12 Months **1/3** No -- Declined -- Don't Know
7.b. Which pests were treated?	**1/3** Cockroaches -- Bees/Wasps **1/3** Ants **1/3** Flies **2/3** Mosquitos -- Termites -- Fleas -- Moths -- Spiders -- Snails -- Rodents -- Declined -- Don't Know	-- Cockroaches -- Bees/Wasps -- Ants -- Flies -- Mosquitos -- Termites -- Fleas -- Moths -- Spiders -- Snails -- Rodents -- Declined -- Don't Know	-- Cockroaches -- Bees/Wasps -- Ants -- Flies -- Mosquitos -- Termites -- Fleas -- Moths -- Spiders -- Snails -- Rodents -- Declined -- Don't Know
8. Was any pesticide spray used around the **outside** of your home from three months prior to becoming pregnant through the end of the child's first year after birth?	**17%** Yes **72%** No **0%** Declined **11%** Don't Know		

8.a. When?	Before Pregnancy? **2/3** Yes **1/3** No -- Declined -- Don't Know	During Pregnancy? **1** Yes [*Mark when*] **1** 1st Trimester **2** 2nd Trimester **2** 3rd Trimester -- Unsure When -- No -- Declined -- Don't Know	First Year? **2/3** 0-6 Months **1/3** 6-12 Months -- No -- Declined -- Don't Know
8.b. Which pests were treated?	**1/3** Cockroaches -- Bees/Wasps **2/3** Ants **2/3** Flies **2/3** Mosquitos -- Termites -- Fleas -- Moths -- Spiders -- Snails -- Rodents -- Declined **1/3** Don't Know	-- Cockroaches **1/3** Bees/Wasps **1/3** Ants **1/3** Flies **1/3** Mosquitos -- Termites -- Fleas -- Moths -- Spiders -- Snails **1/3** Rodents -- Declined **1/3** Don't Know	-- Cockroaches -- Bees/Wasps **1/3** Ants **1/3** Flies **1/3** Mosquitos -- Termites -- Fleas -- Moths -- Spiders -- Snails **1/3** Rodents -- Declined **1/3** Don't Know
9. Did professionals apply any pesticides to kill insects/bugs **inside** your home from three months prior to becoming pregnant through the end of the child's first year after birth?	**10%** Yes **8%** No **0%** Declined **10%** Don't Know		
9.a. When?	Before Pregnancy? **1/2** Yes -- No -- Declined -- Don't Know	During Pregnancy? -- Yes [*Mark when*] -- 1st Trimester -- 2nd Trimester -- 3rd Trimester -- Unsure When -- No -- Declined -- Don't Know	First Year? -- 0-6 Months -- 6-12 Months -- No -- Declined -- Don't Know
9.b. Which pests were treated?	-- Cockroaches -- Bees/Wasps -- Ants -- Flies -- Mosquitos -- Termites -- Fleas -- Moths -- Spiders -- Snails -- Rodents -- Declined -- Don't Know	-- Cockroaches -- Bees/Wasps -- Ants -- Flies -- Mosquitos -- Termites -- Fleas -- Moths -- Spiders -- Snails -- Rodents -- Declined -- Don't Know	-- Cockroaches -- Bees/Wasps -- Ants -- Flies -- Mosquitos -- Termites -- Fleas -- Moths -- Spiders -- Snails -- Rodents -- Declined -- Don't Know
10. Did professionals (exterminators, landscape/ garden service) apply any pesticides to kill bugs **outside** your home from three months prior to becoming pregnant through the end of the child's first year after birth?	**10%** Yes **65%** No **0%** Declined **25%** Don't Know		

10.a. When?	Before Pregnancy? -- Yes **1/2** No -- Declined -- Don't Know	During Pregnancy? -- Yes [*Mark when*] -- 1st Trimester -- 2nd Trimester **1/2** 3rd Trimester -- Unsure When -- No -- Declined -- Don't Know	First Year? -- 0-6 Months **1/2** 6-12 Months -- No -- Declined -- Don't Know
10.b. Which pests were treated?	**1/2** Cockroaches -- Bees/Wasps **1/2** Ants **1/2** Flies **1/2** Mosquitos -- Termites -- Fleas -- Moths -- Spiders -- Snails **1/2** Rodents -- Declined -- Don't Know	-- Cockroaches -- Bees/Wasps -- Ants -- Flies -- Mosquitos -- Termites -- Fleas -- Moths -- Spiders -- Snails **1/2** Rodents -- Declined -- Don't Know	-- Cockroaches -- Bees/Wasps -- Ants -- Flies -- Mosquitos -- Termites -- Fleas -- Moths -- Spiders -- Snails **1/2** Rodents -- Declined -- Don't Know

The next few questions are about air fresheners. The first question is about continuously releasing plug-ins, gels, oils, solids or other types of products that continually or automatically freshen the air. The second question is about air freshener spray products that need to be sprayed by a person. The third question is about air freshener candles and other products that can last for a while after being lit or otherwise activated by a person.

18. Were air fresheners such as plug-ins, gels, oils, solids, or other types of products that continually or automatically freshen the air, used in your home from three months prior to becoming pregnant through the end of the child's first year after birth?	**5%** Yes **75%** No **0%** Declined **20%** Don't Know		
18.a. When?	Before Pregnancy? **1/1** Yes -- No -- Declined -- Don't Know	During Pregnancy? **1/1** Yes [*Mark when*] **1/1** 1st Trimester **1/1** 2nd Trimester **1/1** 3rd Trimester -- Unsure When -- No -- Declined -- Don't Know	First Year? **1/1** 0-6 Months **1/1** 6-12 Months -- No -- Declined -- Don't Know
19. Were air freshener spray products that need to be sprayed by a person used in your home from the time you got pregnant through the end of the child's first year after birth?	**11%** Yes **74%** No **0%** Declined **16%** Don't Know		
19.a. How often on average **during pregnancy**?	-- >3 times per day -- 1-2 times per day **1/2** 1-6 times per week **1/2** < once per week -- Never -- Declined -- Don't know		

19.b. How often on average **during the first year after birth**?	-- >3 times per day -- 1-2 times per day **1/2** 1-6 times per week -- < once per week **1/2** Never -- Declined -- Don't know
20. Were air freshener candles and other products that can last for a while after being lit or otherwise activated by a person used in your home from the time you got pregnant through the end of the child's first year after birth?	**15%** Yes **75%** No **0%** Declined **10%** Don't Know
20.a. How often on average **during pregnancy**?	-- >3 times per day -- 1-2 times per day -- 1-6 times per week **3/3** < once per week -- Never -- Declined -- Don't know
20.b. How often on average **during the first year after birth**?	-- >3 times per day -- 1-2 times per day -- 1-6 times per week **2/3** < once per week -- Never -- Declined 1/3 Don't know
21. Did you wear stain resistant clothing from the time you got pregnant through the end of the child's first year after birth?	**5%** Yes **75%** No **0%** Declined **20%** Don't Know
21.a. How often on average **during pregnancy?**	-- Daily -- 1-6 times per week -- < once per week -- Never -- Declined **1/1** Don't know
21.b. How often on average **during the first year after birth?**	-- Daily -- 1-6 times per week -- < once per week -- Never -- Declined **1/1** Don't know

How often on average did you use each of the following items/products during pregnancy and during the chid's first year after birth?		
24. Mould or mildew cleaners such as Dettol Mould Remover, Harpic or Domestos	**During pregnancy?** **5%** >3 times per day **5%** 1-2 times per day **5%** 1-6 times per week **25%** < once per week **35%** Never **0%** Declined **25%** Don't know	**During child's first year?** **6%** >3 times per day **6%** 1-2 times per day **6%** 1-6 times per week **33%** < once per week **22%** Never **0%** Declined **28%** Don't know
25. Deodorant	**During pregnancy?** **5%** >3 times per day **65%** 1-2 times per day **10%** 1-6 times per week **5%** < once per week **5%** Never **0%** Declined **10%** Don't know	**During child's first year?** **5%** >3 times per day **63%** 1-2 times per day **11%** 1-6 times per week **5%** < once per week **5%** Never **0%** Declined **11%** Don't know
26. Lotion or Cream	**During pregnancy?** **10%** >3 times per day **60%** 1-2 times per day **10%** 1-6 times per week **10%** < once per week **5%** Never **0%** Declined **5%** Don't know	**During child's first year?** **5%** >3 times per day **58%** 1-2 times per day **11%** 1-6 times per week **11%** < once per week **5%** Never **0%** Declined **11%** Don't know
27. Liquid Soap (include both antibacterial and non-antibacterial)	**During pregnancy?** **30%** >3 times per day **50%** 1-2 times per day **5%** 1-6 times per week **0%** < once per week **5%** Never **0%** Declined **10%** Don't know	**During child's first year?** **32%** >3 times per day **42%** 1-2 times per day **5%** 1-6 times per week **0%** < once per week **5%** Never **0%** Declined **16%** Don't know
28. Antibacterial Soaps [*Do **not** include waterless hand gels; include soft soap or bar soaps*]	**During pregnancy?** **10%** >3 times per day **10%** 1-2 times per day **5%** 1-6 times per week **10%** < once per week **35%** Never **0%** Declined **30%** Don't know	**During child's first year?** **11%** >3 times per day **11%** 1-2 times per day **6%** 1-6 times per week **11%** < once per week **28%** Never **0%** Declined **33%** Don't know
29. Hair Gel	**During pregnancy?** **5%** >3 times per day **5%** 1-2 times per day **0%** 1-6 times per week **0%** < once per week **85%** Never **0%** Declined **5%** Don't know	**During child's first year?** **5%** >3 times per day **0%** 1-2 times per day **0%** 1-6 times per week **0%** < once per week **89%** Never **0%** Declined **5%** Don't know
30. Hair Spray	**During pregnancy?** **0%** >3 times per day **0%** 1-2 times per day **0%** 1-6 times per week **0%** < once per week **95%** Never **0%** Declined **5%** Don't know	**During child's first year?** **0%** >3 times per day **0%** 1-2 times per day **0%** 1-6 times per week **0%** < once per week **95%** Never **0%** Declined **5%** Don't know

31. Nail Varnish or Polish Remover	**During pregnancy?** **0%** >3 times per day **0%** 1-2 times per day **5%** 1-6 times per week **21%** < once per week **58%** Never **0%** Declined **16%** Don't know	**During child's first year?** **0%** >3 times per day **0%** 1-2 times per day **5%** 1-6 times per week **21%** < once per week **53%** Never **0%** Declined **21%** Don't know
32. Perfume	**During pregnancy?** **0%** >3 times per day **37%** 1-2 times per day **21%** 1-6 times per week **11%** < once per week **11%** Never **0%** Declined **21%** Don't know	**During child's first year?** **0%** >3 times per day **33%** 1-2 times per day **22%** 1-6 times per week **11%** < once per week **17%** Never **0%** Declined **17%** Don't know
The next questions are about tests for certain exposures the child of interest might have had.		
33. Was the blood of the child of interest tested for lead when he/she was young?	**0%** Yes **50%** No **0%** Declined **50%** Don't Know	

Module O: Occupation and Exposures

Occupational History				
6. Which of the following describes your occupation(s) and the child's biological father's occupation(s) during the period 3 months before pregnancy through the end of the child's first year?				
	Mother			Biological Father
	Before Pregnancy	During Pregnancy	Child's First Year	
A. Agriculture / Farming	**0%**	**0%**	**0%**	**0%**
B. Creative artist or from show business	**0%**	**0%**	**0%**	**5%**
C. Author/ journalist/ linguist	**0%**	**0%**	**0%**	**0%**
D. Hairdresser/ beautician	**0%**	**0%**	**0%**	**0%**
E. Sciences (laboratory technician/researcher)	**0%**	**0%**	**0%**	**0%**
F. Merchant/ salesman/ supermarket teller	**5%**	**5%**	**5%**	**5%**
G. Education professional (teacher / education assistant)	**10%**	**10%**	**10%**	**0%**
H. Lawyer, jurist, judge	**0%**	**0%**	**0%**	**0%**
I. Computer technician (computer engineer, etc.)	**5%**	**5%**	**5%**	**5%**
J. Repair Services / Mechanic / Plumber/ painter/ electrician	**0%**	**0%**	**0%**	**10%**
K. Military	**0%**	**0%**	**0%**	**0%**
L. Driver/ taxi driver/ train driver	**0%**	**0%**	**0%**	**0%**
M. Factory worker	**5%**	**5%**	**5%**	**10%**
N. Fisherman / aquaculture	**0%**	**0%**	**0%**	**0%**
O. Doorman/ housekeeper	**0%**	**0%**	**5%**	**0%**
P. Health professional (doctor / paramedic / nurse / veterinarian / pharmacist)	**5%**	**5%**	**5%**	**5%**
Q. Public security professional (police, firefighter, security)	**0%**	**0%**	**0%**	**5%**
R. Professional catering and hospitality (cook /waiter/bartender etc.)	**0%**	**0%**	**0%**	**15%**
S. Construction Worker	**5%**	**5%**	**5%**	**5%**
T. Work in an office environment	**15%**	**15%**	**15%**	**5%**
U. Other _____	**25%**	**25%**	**20%**	**15%**
V. Not Employed	**10%**	**10%**	**0%**	**0%**
W. Unknown	**0%**	**0%**	**0%**	**0%**
X. Declined	**5%**	**0%**	**0%**	**0%**

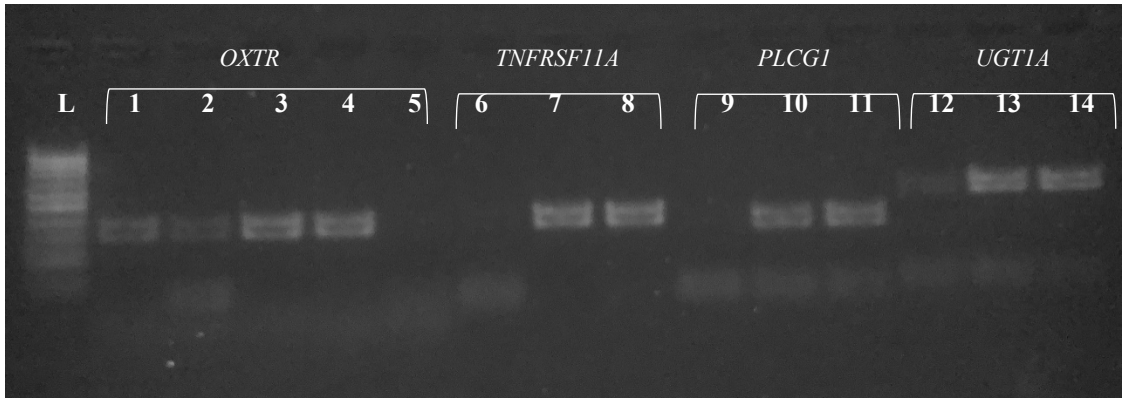
In work or daily life, were you or the child's biological father regularly exposed to any of the following during the 3 months before pregnancy, during pregnancy, or during the child's first year?					
	Biological Parent	Before Pregnancy	During Pregnancy	First Year	
15. Asbestos	Mother	**5%** Yes **75%** No	**5%** Yes **70%** No	**10%** Yes **75%** No	**0%** Declined **20%** Don't Know
	Father	**10%** Yes **70%** No	**10%** Yes **70%** No	**10**% Yes **70%** No	**0%** Declined **20%** Don't Know
16. Chemicals/Acids/Solvents	Mother	**10%** Yes **75%** No	**10%** Yes **75%** No	**10%** Yes **75%** No	**0%** Declined **15%** Don't Know
	Father	**5%** Yes **75%** No	**5%** Yes **75%** No	**5%** Yes **75%** No	**0%** Declined **20%** Don't Know
17. Coal or Stone Dusts	Mother	**5%** Yes **85%** No	**5%** Yes **85%** No	**5%** Yes **85%** No	**0%** Declined **10%** Don't Know
	Father	**5%** Yes **80%** No	**5%** Yes **80%** No	**5%** Yes **80%** No	**0%** Declined **10%** Don't Know
18. Coal Tar/Pitch/Asphalt	Mother	**0%** Yes **85%** No	**0%** Yes **85%** No	**0%** Yes **85%** No	**0%** Declined **15%** Don't Know
	Father	**0%** Yes **85%** No	**0%** Yes **85%** No	**0%** Yes **85%** No	**0%** Declined **15%** Don't Know
19. Diesel Engine Exhaust	Mother	**10%** Yes **75%** No	**10%** Yes **75%** No	**10%** Yes **75%** No	**0%** Declined **15%** Don't Know
	Father	**10%** Yes **55%** No	**10%** Yes **60%** No	**10%** Yes **60%** No	**0%** Declined **25%** Don't Know
20. Dyes	Mother	**5%** Yes **80%** No	**0%** Yes **85%** No	**0%** Yes **85%** No	**0%** Declined **15%** Don't Know
	Father	**10%** Yes **75%** No	**5%** Yes **80%** No	**5%** Yes **80%** No	**0%** Declined **15%** Don't Know
21. Formaldehyde	Mother	**0%** Yes **80%** No	**0%** Yes **80%** No	**0%** Yes **80%** No	**0%** Declined **20%** Don't Know
	Father	**0%** Yes **80%** No	**0%** Yes **80%** No	**0%** Yes **80%** No	**0%** Declined **20%** Don't Know
22. Petrol Exhaust	Mother	**20%** Yes **75%** No	**20%** Yes **75%** No	**20%** Yes **75%** No	**0%** Declined **5%** Don't Know
	Father	**25%** Yes **65%** No	**20%** Yes **70%** No	**20%** Yes **70%** No	**0%** Declined **10%** Don't Know
23. Pesticides/Herbicides	Mother	**5%** Yes **75%** No	**5%** Yes **75%** No	**0%** Yes **80%** No	**0%** Declined **20%** Don't Know
	Father	**15%** Yes **70%** No	**15%** Yes **70%** No	**10%** Yes **75%** No	**0%** Declined **15%** Don't Know
24. Textile Fibres/Dusts	Mother	**5%** Yes **70%** No	**5%** Yes **75%** No	**5%** Yes **70%** No	**0%** Declined **20%** Don't Know
	Father	**15%** Yes **55%** No	**15%** Yes **55%** No	**15%** Yes **55%** No	**0%** Declined **30%** Don't Know
25. Wood Dust	Mother	**5%** Yes **85%** No	**0%** Yes **80%** No	**0%** Yes **85%** No	**0%** Declined **10%** Don't Know
	Father	**0%** Yes **80%** No	**0%** Yes **80%** No	**0%** Yes **80%** No	**0%** Declined **20%** Don't Know
26. X-rays/Radioactive Materials	Mother	**10%** Yes **80%** No	**5%** Yes **85%** No	**0%** Yes **90%** No	**0%** Declined **10%** Don't Know
	Father	**5%** Yes **70%** No	**5%** Yes **70%** No	**5%** Yes **70**% No	**0%** Declined **25%** Don't Know
27. Varnish/Lacquer	Mother	**10%** Yes **75%** No	**5%** Yes **85%** No	**10%** Yes **75%** No	**0%** Declined **15%** Don't Know
	Father	**10%** Yes **70%** No	**10%** Yes **70%** No	**10%** Yes **70%** No	**0%** Declined **20%** Don't Know

Module I: Instrument Rating

Questions about this questionnaire	
1. Please Rate the overall questionnaire length	**0%** Much too short **0%** A little too short **10%** Acceptable **45%** A little too long **45%** Much too long
2. How sure were you about your answers to questions on:	
a) Medical history	**68%** Very Sure **16%** Somewhat Sure **16%** Somewhat Unsure **0%** Very Unsure
b) Breastfeeding	**85%** Very Sure **10%** Somewhat Sure **5%** Somewhat Unsure **0%** Very Unsure
c) Diet	**61%** Very Sure **17%** Somewhat Sure **22%** Somewhat Unsure **0%** Very Unsure
d) Vitamins/Supplements	**63%** Very Sure **16%** Somewhat Sure **21%** Somewhat Unsure **0%** Very Unsure
e) Lifestyle/Substance Use	**53%** Very Sure **42%** Somewhat Sure **5%** Somewhat Unsure **0%** Very Unsure
f) Your Home	**84%** Very Sure **16%** Somewhat Sure **0%** Somewhat Unsure **0%** Very Unsure
g) Your Environment	**42%** Very Sure **53%** Somewhat Sure **5%** Somewhat Unsure **0%** Very Unsure
h) Occupations/Exposures	**47%** Very Sure **42%** Somewhat Sure **11%** Somewhat Unsure **0%** Very Unsure
3. How sure were you about your answers to questions on timing for:	
i) Medical history	**67%** Very Sure **28%** Somewhat Sure **6%** Somewhat Unsure **0%** Very Unsure
j) Breastfeeding	**72%** Very Sure **17%** Somewhat Sure **11%** Somewhat Unsure **0%** Very Unsure
k) Diet	**72%** Very Sure **11%** Somewhat Sure **17%** Somewhat Unsure **0%** Very Unsure
l) Vitamins/Supplements	**59%** Very Sure **29%** Somewhat Sure **12%** Somewhat Unsure **0%** Very Unsure
m) Lifestyle/Substance Use	**61%** Very Sure **33%** Somewhat Sure **6%** Somewhat Unsure **0%** Very Unsure
n) Your Home	**83%** Very Sure **11%** Somewhat Sure **6%** Somewhat Unsure **0%** Very Unsure
o) Your Environment	**50%** Very Sure **39%** Somewhat Sure **11%** Somewhat Unsure **0%** Very Unsure
p) Occupations/Exposures	**59%** Very Sure **29%** Somewhat Sure **12%** Somewhat Unsure **0%** Very Unsure
Please indicate how much you agree or disagree with the following statements.	
4. The questionnaire instructions were clear.	**68%** Agree **21%** Somewhat Agree **5%** Neutral **5%** Somewhat Disagree **0%** Disagree
5. Overall, I felt sure of my answers to the questions.	**53%**Agree **37%** Somewhat Agree **11%** Neutral **0%** Somewhat Disagree **0%** Disagree
6. The questions asked were important.	**61%**Agree **28%** Somewhat Agree **0%** Neutral **11%** Somewhat Disagree **0%** Disagree
7. The length of the section on **Medical History** was appropriate.	**59%**Agree **29%** Somewhat Agree **0%** Neutral **12%** Somewhat Disagree **0%** Disagree
8. The length of the section on **Breastfeeding** was appropriate.	**67%**Agree **33%** Somewhat Agree **0%** Neutral **0%** Somewhat Disagree **0%** Disagree
9. The length of the section on **Diet** was appropriate.	**50%**Agree **33**% Somewhat Agree **11%** Neutral **6%** Somewhat Disagree **0%** Disagree
10. The length of the section on **Vitamins/Supplements** was appropriate.	**47%**Agree **41%** Somewhat Agree **12%** Neutral **0%** Somewhat Disagree **0%** Disagree
11. The length of the section on **Lifestyle/Substance Use** was appropriate.	**67%**Agree **22%** Somewhat Agree **11%** Neutral **0%**Somewhat Disagree **0%** Disagree

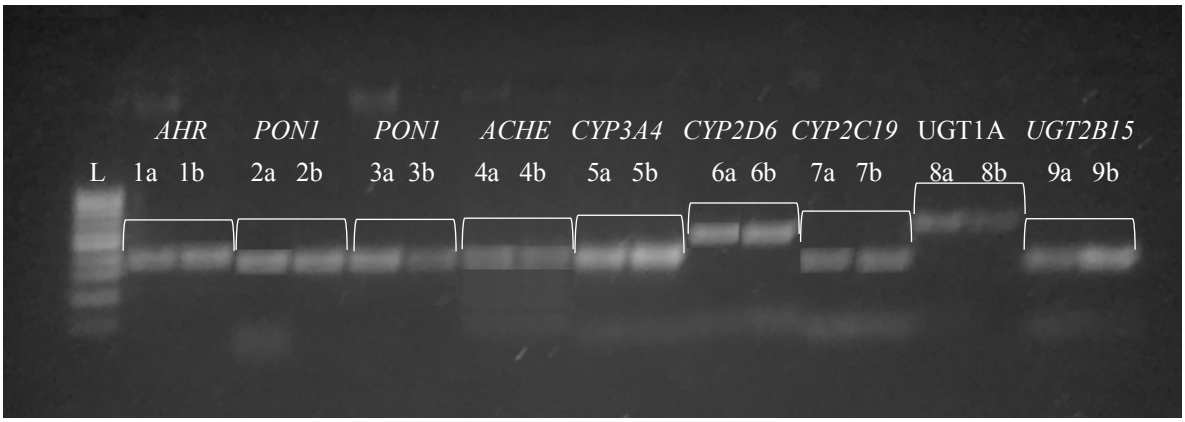
12. The length of the section on **Home Environment** was appropriate.	**61%** Agree **22%** Somewhat Agree **17%**Neutral **0%**Somewhat Disagree **0%** Disagree
13. The length of the section on **Renovations and Chemical Exposures** was appropriate.	**56%**Agree **22%** Somewhat Agree **22%** Neutral **0%** Somewhat Disagree **0%** Disagree
14. The length of the section on **Occupations and Exposures** was appropriate.	**44%**Agree **22%** Somewhat Agree **33%** Neutral **0%** Somewhat Disagree **0%** Disagree
15. The ELEAT questionnaire was easy to find on the webpage.	**79%**Agree **0%** Somewhat Agree **14%** Neutral **7%** Somewhat Disagree **0%** Disagree
16. I had no difficulties logging into the online survey.	**79%**Agree **7%** Somewhat Agree **7%** Neutral **7%** Somewhat Disagree **0%** Disagree
17. It was easy to navigate through questions on the survey.	**93%**Agree **7%** Somewhat Agree **0%** Neutral **0%** Somewhat Disagree **0%** Disagree
18. It was easy to understand the instructions given for completing the questionnaire online.	**80%**Agree **13%** Somewhat Agree **7%** Neutral **0%** Somewhat Disagree **0%** Disagree
19. I liked the overall look of the website and the online survey.	**47%**Agree **29%** Somewhat Agree **24%** Neutral **0%** Somewhat Disagree **0%** Disagree
20. Other Comments?	_____ _____ _____

Attachment S5



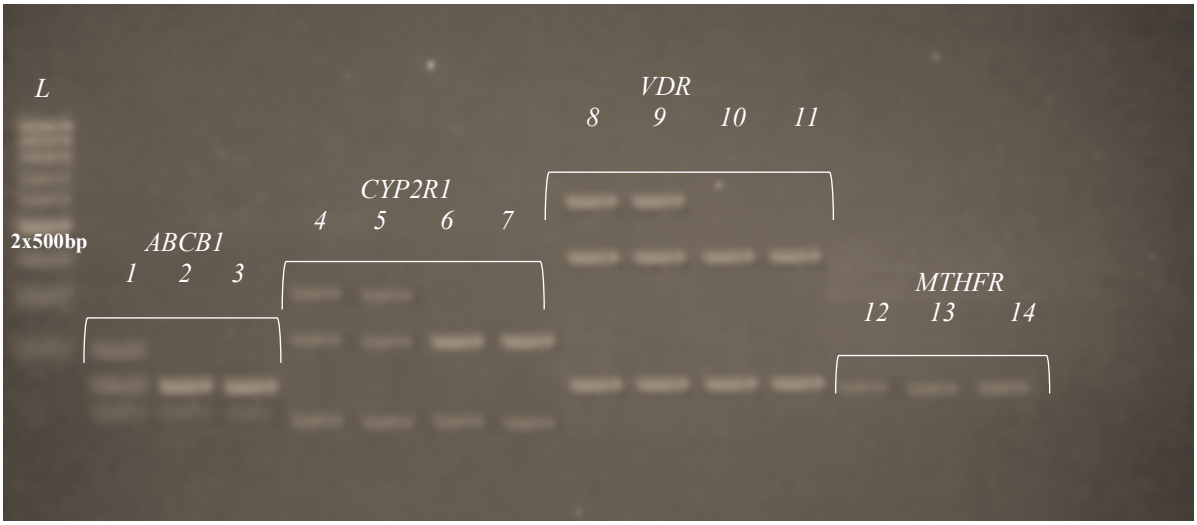
Attachment S6: **1.8% electrophoresis gel containing amplification fragments for the polymorphisms analyzed by Sanger Sequencing for medications taken by the mothers.** Lanes 1-5 (312bp) are for the 5 subjects analyzed for rs2268498 polymorphism of *OXTR,* lanes 6-8 (332bp) are for the 3 subjects analyzed for rs1805034 polymorphism of *TNFRSF11A,* lanes 9-11 (306bp) are for rs2228246 polymorphism of *PLCG1*; lanes 12-14 (487bp) are for rs1042640 polymorphism of *UGT1A*. L stands for 100bp DNA Ladder (Bioron).

Attachment S6



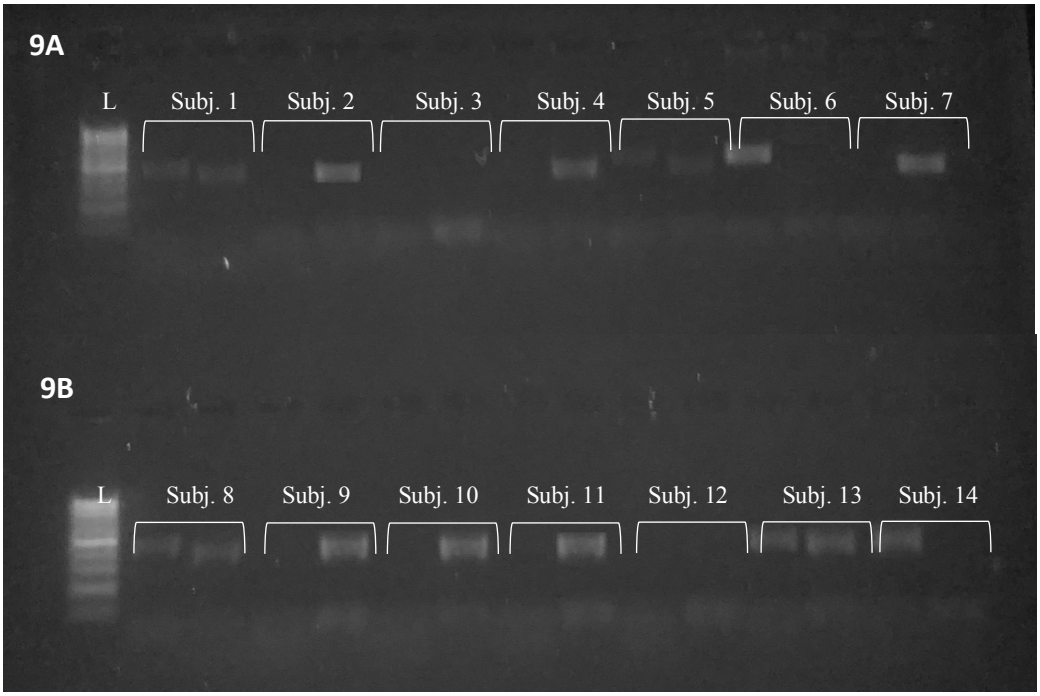
Attachment S7: **1.8% electrophoresis gel containing amplification fragments for the polymorphisms analyzed by Sanger Sequencing in all 14 subjects.** Two examples of the obtained fragments are shown for each polymorphism. Lanes 1a and 1b (389bp) – rs4410790 polymorphism for *AHR*; lanes 2a and 2b (337bp) – rs662 polymorphism for *PON1*; lanes 3a and 3b (332bp) – rs854560 polymorphism for *PON1*; lanes 4a and 4b (316bp) – rs2571598 polymorphism for *ACHE*; lanes 5a and 5b – rs2749574 polymorphism for *CYP3A4* (329bp); lanes 6a and 6b (443bp) – CYP2D6*4 and CYP2D6*6 (both are present in a fragment amplified through the same pair of primers) for *CYP2D6*; lanes 7a and 7b (301bp) – CYP2C19*2 for *CYP2C19*; lanes 8a and 8b (487bp) – two polymorphisms for UGT1A (we were unable to sequence); lanes 9a and 9b (267bp) – rs1902023 polymorphism for *UGT2B15* gene. L stands for 100bp DNA Ladder (Bioron).

Attachment S7:



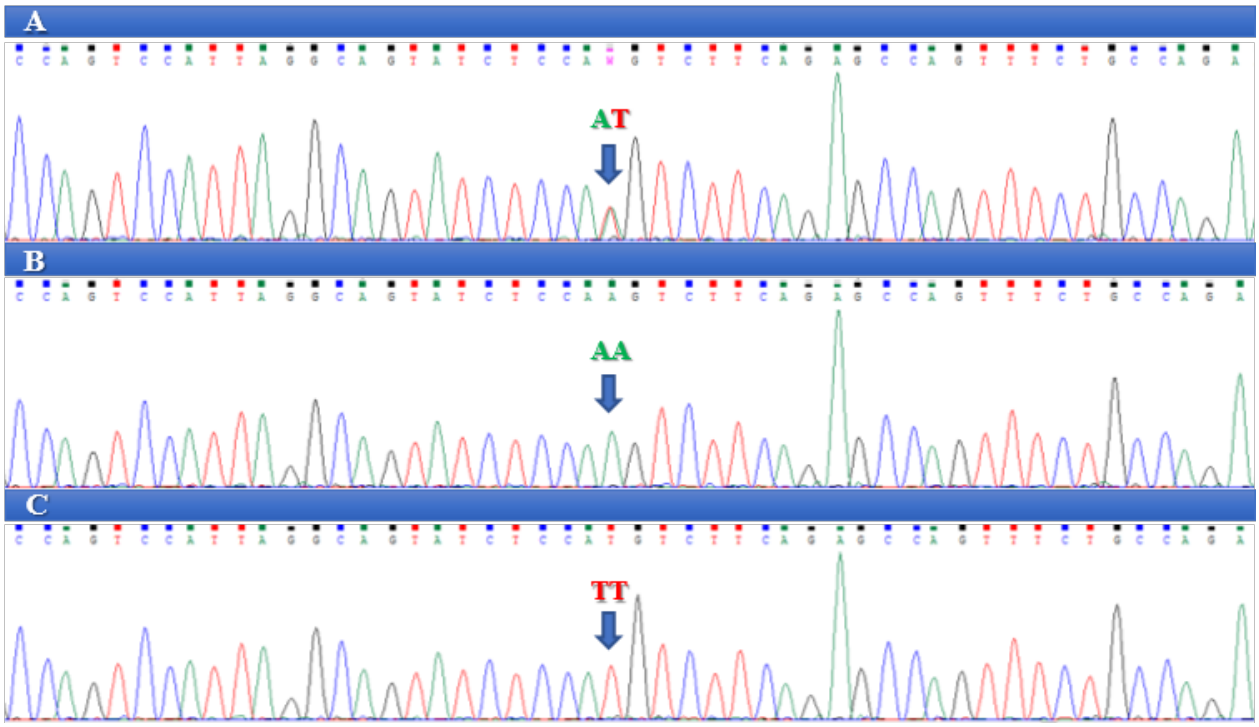
Attachment S8: **3% electrophoresis gel with examples of genotype patterns for each polymorphism assessed through RFLP.** Lanes 1, 2 and 3 are for rs1045642 polymorphism of *ABCB1* (lane 1 corresponds to heterozygous GA subject 10 that displays three bands of 206bp, 130bp and 76bp and lanes 2 and 3 correspond to homozygous GG subjects 4 and 8 which display two bands of 130bp and 76bp). Lanes 4 to 7 are for rs10741657 polymorphism of *CYP2R1* (lanes 4 and 5 correspond to heterozygous AG subjects 7 and 9, displaying three bands of 300bp, 248bp and 52bp and lanes 6 and 7 correspond to homozygous AA subjects that display two bands of 248 and 52bp). Lanes 8 to 11 are for rs731236 polymorphism of *VDR* (lanes 8 and 9 correspond to heterozygous TC subjects 2 and 9, which display three bands of 630, 425 and 205bp, while lanes 10 and 11 are for homozygous TT subjects that display two bands of 425 and 205bp). Lanes 12, 13 and 14 are for rs1801133 polymorphism of *MTHFR*, of which all 14 subjects where homozygous CC (shown are bands for subjects 2, 9 and 10). L stands for 100bp DNA Ladder (Bioron).

Attachment S8



Attachment S9: **1.8% electrophoresis gel with genotype pattern of *GSTM1* for the 14 assessed subjects.** As stated in methods section, two pairs of primers were used for each subject resulting in two lanes: if there is deletion of *GSTM1* a band of 433bp appears in the first lane, while the presence of *GSTM1* corresponds to a 379bp in the second lane. Heterozygous subjects (*GSTM1*+/-) will have both bands, while homozygous *GSTM1*-/- or *GSTM1*+/+ will have a band only in the first or second lane, respectively. Subjects 2, 4, 7, 9, 10 and 11 are homozygous for the presence of the gene (*GSTM1* +/+). Subjects 6 and 14 are homozygous for the deletion of the gene (*GSTM1* -/-). Subjects 1, 5, 8 and 13 are heterozygous (*GSTM1* +/-). No pattern was obtained for subjects 3 and 12. L stands for 100bp DNA Ladder (Bioron).

Attachment S9



Attachment S10: **Partial sequencing electropherogam obtained for the genotyping of PON1 regarding rs854560 polymorphism.** A – Heterozygous (AT) subject 9; B – Homozygous AA subject 11; C – Homozygous TT subject 4